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THE RELATIVE PATHOGENICITY OF SOME SALMONELLA SEROTYPES FOR CHICKS

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Received 21 Dec 1959

A GREAT deal of evidence has been accumulated to show that Salmonella serotypes can be isolated from flocks of chicks which were experiencing severe mortality. Very little work has been done, except with *Salmonella pullorum* and *Salmonella gallinarum*, to show the actual mortality that serotypes may cause when fed to chicks. Milner and Shaffer[2] and Shaffer, *et al.*[4] have infected chicks orally with known doses of various Salmonella species as a measure of the infective ability of the organisms as determined by the ability to recover the organisms. They have some data on the mortality following infection. Gwatkin and Grinewitch[1] and Seiburth and Johnson[3] have reported on the mortality caused by *Salmonella typhimurium* when given orally to chicks. Since it is sometimes difficult to convince a poultryman or hatcheryman that these organisms can cause mortality, the following experiments were undertaken.

MATERIALS AND METHODS

Purdue, random-mated, closed flock, Single Comb White Leghorn chicks with an average inbreeding coefficient of 20 percent were used in all experiments. They were housed under

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nearly ideal brooding conditions in two Oakes battery brooders with five tiers in each battery. Each tier was separated from the one below by a sheet of plywood which extended six inches beyond the batteries on all four sides. This was done in an attempt to minimize the spread of infection from one lot to another. The groups of chicks not exposed or with lower dosage were placed above those with greater dosages. The birds were fed a pelleted commercial breeder ration which contained no antibiotics, coccidiostats or other drugs. Water and feed were kept before the birds at all times. No record of feed consumption was maintained. The birds were not weighed, with the exception that those birds exposed to *Salmonella enteritidis* were weighed individually at 4 weeks of age. All birds were kept for 4 weeks.

All chicks, except the controls, were orally exposed at one day of age, with Salmonella isolates from chickens or turkeys submitted to Purdue Animal Disease Diagnostic Laboratory. The organisms had been maintained on tryptose agar (Difco) for periods varying from 4 to 20 months. The organisms were grown in nutrient broth for 48 hours. The number of organisms per ml was determined by diluting and plating the broth culture on a series of tryptose agar plates which were incubated for 48 hours. The colonies were counted and the number of organisms were determined by calculation. The broth culture was diluted to give 100,000,000 organisms in 0.1 ml. Then ten fold dilutions were made until the greatest dilutions theoretically contained one organism per 0.1 ml. Each 0.1 ml dose was put into a number four gelatin capsule and this was forced into the crop of each day-old chick. In some of the experiments the highest dosage, because of the volume of broth involved, had to be given with a pipette.

In each experiment, except the first one with *Salmonella anatum*, 200 day-old chicks were used and divided into ten groups of 20. In each experiment with each serotype, except *Salmonella anatum* first trial, 20 chicks were not inoculated. The other nine groups received a dosage of one, ten, and in multiples of ten up to 10^8 organisms. In the first experiment with *Salmonella anatum* where the wire floors were retained

in the batteries, there were ten non-exposed chicks, 15 chicks in the groups receiving one and ten organisms, 28 chicks in the group receiving 1000 organisms, and 17 in the group receiving 100,000 organisms. There were 20 chicks in each of the other five groups in this one trial.

One experiment was conducted with each of the following: *Salmonella typhimurium, Salmonella enteritidis, Salmonella reading, Samonella heidelberg, Salmonella indiana.* Two experiments were conducted with each of *Salmonella seftenberg* and *Salmonella anatum.* In the case of *Salmonella seftenberg,* the inoculum for the second trial was prepared from a culture isolated from a dead chick from the first trial. In the second trial with *Salmonella anatum,* a duplicate culture of that used in the first trial was employed. All original cultures were isolated at the Animal Disease Diagnostic Laboratory at Purdue University and were typed by the Salmonella Typing Center at Chamblee, Georgia. All chicks which died were cultured by inoculating selenite (Difco) tubes with livers and unabsorbed yolks if present. After 18 hours at 37 C incubation and after another 24 hours at room temperature, transfers were made from the selenite broths to brilliant green agar (Difco). Only those chicks from which bacteria were recovered which gave carbohydrate (lactose, sucrose, dextrose, maltose, and mannitol) motility, indol, and urease reactions typical for paratyphoid were recorded as deaths due to the particular serotype. Originally it was proposed that all recovered organisms would be typed using specific typing sera. However, it was found impractical to type all recoveries so only a limited number in each experiment were typed with Salmonella typing sera.

RESULTS

The mortality which occurred during the 4 weeks following *per os* inoculation with the various serotypes is recorded in tables 1, 2, and 3. There were some deaths which were not recorded in these tables. In the experiments with *Salmonella seftenberg* and *Salmonella heidelberg* there were two birds in each experiment which died and bacteriological cultures were negative. There was one death without the recovery of *Sal-*

monella anatum in the *Salmonela anatum* experiment where the wire floors were removed. In the *Salmonella anatum* experiment with the wire floors and in the *Salmonella reading* experiment there were in each case three deaths due to omphalitis where the appropriate organisms were not recovered.

TABLE I

Mortality in chicks due to selected Salmonella species following *per os* exposure*

No. of bacteria per chick	Salmonella Species						
	A	B	C	D	E	F	G
None		2			1		0/15
1	11	7	4	3		1	0/10
10	13	4	3		3		0/20
10^2	17	2	3				0/15
10^3	17	2	2	3	1		1/28
10^4	18	1	1		2		0/20
10^5	15	1	3	2		1	2/17
10^6	16	4	4	1			1/20
10^7	19	4	3			2	0/20
10^8	20	7	2	4	2	1	0/20
Totals	146	34	25	13	9	5	4

A—*S. typhimurium*
B—*S. indiana*
C—*S. enteritidis*
D—*S. heidelberg*
E—*S. seftenberg*
F—*S. reading*
G—*S. anatum*

*Twenty chicks were inoculated in each dosage group with the exceptions recorded under G. In column G the numerator represents the deaths and the denominator the number inoculated.

In the experiment with *Salmonella enteritidis* where the chicks were weighed individually at 4 weeks, there was no significant difference in weights between the ten groups.

DISCUSSION

The mortality produced by the oral administration of the seven serotypes as recorded in table 1 indicates the relative mortality caused by the various species under good brooding conditions with small numbers of chicks in each group. One would not expect to be able to reproduce the exact mortality shown, but more likely only a relative mortality between the various serotypes.

Salmonella typhimurium produced a relatively high mor-

TABLE II

A comparison of the mortality in chicks following *per os* exposure to *Salmonella seftenberg* before and after chick passage*

No. of bacteria per chick	Before chick passage	After chick passage
None	1	
1		
10	3	4
10^2		2
10^3	1	1
10^4	2	2
10^5		
10^6		1
10^7		4
10^8	2	2
Totals	9	16

*Twenty chicks were inoculated in each dosage group.

TABLE III

A comparison of the mortality in chicks following *per os* exposure to *Salmonella anatum* with and without the use of wire floors in the Brooders*

No. of bacteria per chick	Wire Floors	Wire Floors Removed
None	0/15	
1	0/10	2
10	0/20	1
10^2	0/15	
10^3	1/28	3
10^4	0/20	1
10^5	2/17	2
10^6	1/20	
10^7	0/20	1
10^8	0/20	
Totals	4	10

*Twenty chicks were inoculated in each dosage group with the exceptions noted in the first experiment. Here the numerator represents the deaths and the denominator the number inoculated.

tality of 80 percent. As reported by Seiburth[3], mortality did not seem to be related to dosage, except that the groups receiving one and ten organisms had considerably lower mortality than those receiving the higher dosages. With the other

species there was apparently no relationship between dosage and mortality.

Salmonella heidelberg has been important because of its interference with the pullorum testing program. This organism produced a nine percent mortality in this experiment which indicates that it could cause mortality in chicks. In Indiana, *Salmonella indiana* has been isolated from breeders and chicks. It, like *Salmonella heidelberg*, can interfere with the pullorum testing and the 34 percent mortality recorded here indicates that it could be a serious problem in chicks.

In table 2, the deaths before and after one chick passage of *Salmonella seftenberg* are recorded. After passage there were 16 deaths as compared with nine in the first trial. This difference is not significant at the five percent level, and can only be interpreted as the difference that may occur in this type of experiment.

The mortality caused by *Salmonella anatum* in the two experiments is shown in table 3. In the first one, the wire floors limited the exposure to the droppings, whereas in the second trial with the wire floors removed the chicks walked in their own feces. With the wire floors the mortality was four chicks; without the wire floors the mortality was ten chicks. This difference is significant at the five percent level. However, if the birds become infected from the droppings, one might expect the increased mortality to occur later than in the group on wire floors. This did not occur. Therefore, the significance of this difference may be questioned.

SUMMARY

Seven different Salmonella serotypes were given orally in graduated dosages to inbred Single Comb White Leghorn day-old chicks. *Salmonella typhimurium, Salmonella indiana, Salmonella enteritidis, Salmonella heidelberg* and *Salmonella reading* were each used in separate experiments. *Salmonella seftenberg* and *Salmonella reading* were each used in two separate experiments. All seven serotypes, under nearly ideal brooding conditions in batteries, produced mortality varying from about 2 percent (*Salmonella anatum*) to 80 percent (*Sal-*

monella typhimurium) when given to day-old chicks. One immediate chick passage of *Salmonella seftenberg* did not significantly increase the mortality caused by this serotype. With *Salmonella anatum*, the exposure of the infected chicks to their own feces significantly increased the mortality but did not extend the period of mortality.

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HATCHERY SPREAD OF PULLORUM DISEASE THROUGH DEBEAKING

By R. E. JACOBS, M. N. FRAZIER, and M. E. TOURTELLOTTE

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NUMEROUS reports have been made on the transmission of pullorum disease, the most common means being via eggs from an infected carier (Rettger and Plastridge).[6] The

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disease is also spread by contact (Edwards and Hull[2]), infected vaccines (Gorrie,[4] Burr *et al.*[1]) and flies (Gwatkin and Mitchell[5]). The experimental transmission of *Salmonella pullorum* by chick sexing instruments was reported by Gordon *et al.*[3]

Several outbreaks of pullorum disease in chicks were traced to a common source, with debeaking instruments being a possible source of infection. The experimental transmission of pullorum disease by a debeaking instrument was demonstrated and a suggested means of sanitization is proposed.

CASE HISTORY

Salmonella pullorum was isolated from a consignment of chicks which had been hatched in Massachusetts and brought to a Connecticut hatchery for debeaking. Chicks from the same hatch that were not debeaked and went to another farm failed to present evidence of pullorum disease. During the preceding several months *S. pullorum, S. heidelberg* and *S. oranienburg* had been isolated from chicks which had been hatched and debeaked at the Connecticut hatchery. A visit to the hatchery showed that, other than wirebrushing of the debeakers, no program of sanitization was employed between processing chicks from different source flocks. This observation suggested that the debeakers became contaminated by debeaking chicks affected with pullorum disease, and remained as foci of infection for subsequent clean chicks.

MATERIALS AND METHODS

Preparation of inoculum. A pool of triturated heart, liver, spleen, gall bladder, and intestine from infected day-old chicks (accession number A79441-50) which exhibited typical lesions of pullorum disease and from which *S. pullorum* was isolated later, was used as the inoculum for contamination of the debeaker.

Debeaker preparation. A Lyons Super Debeaker* (electrical type) was used. For the experiments, the bar at the bottom of the debeaker was contaminated with the infectious

*Manufactured by the Lyons Rural Electric Company, San Diego, California

material. For determination of the efficiency of sterilization, six sites, namely the top, right side, right bar, porcelain block, lower front bar and hole, and left side of the left fin, were inoculated (figure 1).

Media. Heart infusion broth plus 0.5 percent glucose and 1.25 ml of a 1.6 percent alcoholic suspension of phenol red per liter were used for detection of growth of the organisms in the experiment on the determination of the thermal death point of *S. pullorum* and the test organism, *Escherichia coli*, for field use. Tryptose agar slants were used for recovery of organisms in the experiment on determination of the efficiency of sterilization of the debeakers. Tryptose agar slants were again used plus selenite broth and SS agar for isolation of *S. pullorum*.

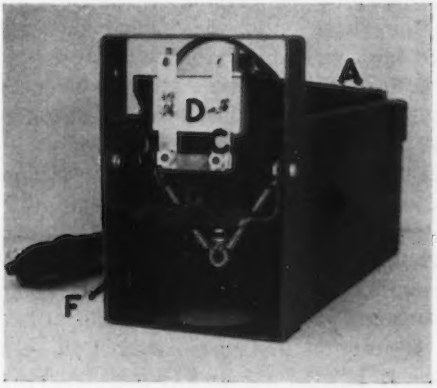

Fig. 1. Debeaker used with swabbing sites indicated: A, top; B, right side; C, right bar; D, porcelain block; E, lower front bar and hole; F, left side of left fin.

Experimental chicks. The 100 one-day-old chicks used in the experiment were from a flock which had recently been tested and found to be pullorum free. For transmission experiments, three lots of chicks were debeaked; group 1, 10 chicks; group 2, 20 chicks and group 3, 30 chicks. The bar at the bottom of the debeaker was swabbed with inoculum between each group. Inoculum was pipetted directly into the

crops of 10 non-debeaked chicks, group 4. Group 5 consisted of 30 non-debeaked, non-infected controls.

Selection of test organism. To avoid the use of viable cultures of *S. pullorum* at the hatchery for determination of the efficiency of sanitization procedures, a relatively innocuous organism test, *Escherichia coli* A88324, was chosen with approximately the same thermal death point as *S. pullorum*. Before determination of the thermal death point was attempted, cultures of *E. coli* and the *S. pullorum* control, A77324, were transferred daily for 5 consecutive days in heart infusion broth and shaken for 15 minutes. Sterile, soft glass capillary tubes, approximately 1 mm in internal diameter, and 50 to 60 mm in length were picked up with flamed forceps, one end dipped into the broth culture which had been poured into a petri dish. One-third to one-half of the tube was filled by capillary action. Both ends were sealed and different tubes were exposed in a water bath, to temperatures from 44 to 58 C for 10 minutes. The exposure temperature was varied by increments of two degrees. The tube was broken aseptically and the tube and its contents were added to sterile broth. The thermal death point was the lowest temperature that killed the exposed culture in the allotted time.

Laboratory sterilization of debeakers. Test sites on the debeakers were swabbed with cultures of *S. pullorum* and *E. coli*. The machines were exposed to dry heat temperatures in an electric oven at temperatures of 82.2, 101.6, 107.2 and 168.3 C for 10 minutes and to moist heat in an autoclave at a temperature of 121 C for 5 and 10 minutes, respectively.

Field sanitization of debeakers. The hatchery provided a 50 gallon metal drum, 36 inches high and 23 inches in diameter with two openings, one approximately 10 inches from the bottom for the exit of condensed steam. A metal rack 17 inches from the bottom was fitted horizontally for placement of the debeakers. There was a fitted cover for the top. A 24 hour culture of the test organism, *E. coli*, was used to inoculate the six previously mentioned test sites and trials were made with (trial 1 and 2) and without (trial 3) the fumigant, one

ounce of formalin activated by one teaspoon (4 grams) of $KMnO_4$. In trial 1, adequate steam pressure could not be obtained. For succeeding trials 2 and 3, the steam pressure had been restored. Prior to the exposure period, the steam was turned on for 10 minutes to preheat the chamber. The exposure time was 10 minutes for trial 1 and 20 minutes for trials 2 and 3.

RESULTS

Recovery of S. pullorum from experimental chicks. All internal organs of the chicks in group 1 were apparently normal. In group 2, two chicks showed heart nodules, one a thickening of the intestine, one had inflammation of the spleen and the remainder were normal. Air sac involvement was noted in nine chicks from group 3 and three isolations were made from 30 chicks (table 1). None of those with air sac lesions were positive for *S. pullorum*. One chick from group 4 showed nodules on the heart, gizzard and pancreas. *S. pullorum* was isolated from a second chick showing nodules on the pancreas. A third showed only nodules on the pancreas. The other four chicks from which *S. pullorum* was isolated had no nodules. Group 5, had only one abnormal chick with a hemorrhagic condition of the intestine.

The rapid plate test was negative for groups 1, 2, and 3. Group 4 had eight reactors out of ten chicks. *S. pullorum* was isolated from the two chicks which were serologically negative. Group 5 had one chick with a 1+ fine type of reaction.

TABLE I

Experimental infection of clean chicks with *S. pullorum* by debeaking with a machine contaminated with known, infective material

Group	Chicks per group	Method of inoculation	Recovery of organism	Percent recovery of organism
I	10	Contaminated debeaker	0	0
II	20	Contaminated debeaker	0	0
III	30	Contaminated debeaker	3	10
IV	10	Via crop	5	50
V	30	Uninoculated controls	0	0

Comparison of heat lability of E. coli and S. pullorum. Five trials were done to determine the thermal death point of our strains of *S. pullorum* and *E. coli.* Both organisms survived temperatures in a water bath at 54 C for 10 minutes but not 56 C.

Effectiveness of laboratory sterilization of debeakers. A dry heat temperature of 82.2 C was ineffective against *S. pullorum* or *E. coli.* Growth in broth inoculated with swabs from the test sites of the debeaker after the period of exposure was equal to the growth of the non-heated control. At a temperature of 107.2 C, *S. pullorum* survived on all but one area, the right front bar. Recontamination with *E. coli* without prior heating and then holding the temperature at 101.6 C allowed survival of organisms in four areas. *S. pullorum* and *E. coli* survived on one, *E. coli* alone on one and *S. pullorum* alone on two areas. The agent was not killed by two exposures to heat at this temperature. A temperature of 168.3 C, sterilized every area, but caused considerable damage to the machine.

Moist heat at both exposure times at a temperature of 121 C and 15 pounds pressure completely sterilized the debeaker as evidenced by failure to recover the organisms. Machine damage by this method was negligible.

Effectiveness of field sanitization of debeakers. Faulty boiler operation produced temperatures below 100 C in trial 1. Test organisms were recovered from all of the six sites. No test organisms were recovered in trial 2 when the temperature had been maintained at 100 C for the 20 minute exposure period; however viable organisms identified as *Gaffkya sp.* were isolated. Results of trial 3 containing fumigant were similar to those of trial 2.

DISCUSSION

The results of this experiment, three isolations of *S. pullorum* in sixty attempts, showed that the causative agent of pullorum disease could be transmitted when pullorum clean chicks were debeaked with a machine contaminated with known, infective material.

Incrimination of a contaminated debeaker as a mode of

transmission for the agent of pullorum disease clearly indicated the need for a sanitizing program. Thus, at the end of each day's operation or between chicks from different source flocks, machines should be sanitized. The sanitization program specifies thorough cleansing of the machine and exposure at a temperature and time which would be well above the thermal death point of enteric organisms which are of pathogenic significance.

SUMMARY

The debeaking procedure as a method of transmission of pullorum disease has been accomplished experimentally by contaminating the machine with known, infective material. Isolations of *S. pullorum* from three of 60 debeaked chicks showed that pullorum disease can be transmitted to pullorum clean chicks through the debeaking process.

The thermal death point of a strain of *E. coli* and *S. pullorum*, was determined to be 54 C. The effective dry heat temperature was 168.3 C. This temperature, however, was injurious to the machine. Moist heat (steam) for 20 minutes without the addition of a fumigant, that produced a temperature of 100 C, killed the test organisms with minimal damage to the equipment.

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STUDIES ON AVIAN ENCEPHALOMYELITIS. III IMMUNE RESPONSE TO BETA-PROPIOLACTONE INACTIVATED VIRUS*†

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THE control of avian encephalomyelitis (AE) by immunization of young chickens has been the topic of several recent papers.[2,3,4,5] Wing-web and intramuscular vaccines, though capable of creditably immunizing birds, have been more pathogenic than is desirable. Oral administration of virus has been disappointing because of the extremely high dosage necessary to effect an adequate immunity.[2] In 1959, Schaaf reported the development of a beta-propiolactone (BPL) inactivated AE vaccine for use when there is a risk of spreading live virus to susceptible breeders.[4] Such a vaccine would also obviate the problem of pathogenicity and post-vaccination reaction. A vaccine containing roughly 1,500,000 CLD_{50}/ml, 6.25 percent aluminum hydroxide, and with a pH of 7.4 proved to be the most effective in the experiments conducted by Schaaf. He found that the inclusion of the adjuvant

* Contribution No. 1224 of the Department of Veterinary Science, Massachusetts Agricultural Experiment Station, Amherst.
† This work was supported in part by a grant from DeKalb Agricultural Association, Inc., DeKalb, Illinois.

had a marked effect on increasing the proportion of immune birds following the use of his BPL vaccine. The pH (4.4 to 7.4) of the vaccine, on the other hand, exerted little or no effect on subsequent immunity.

The data submitted here is intended not only to confirm Schaaf's report on the efficacy of a BPL inactivated vaccine but also to provide additional information regarding effective virus dosage and serological responses following vaccination.

MATERIALS AND METHODS

Virus and vaccine preparation. The virus used in these trials was embryo-adapted.[1] Brain material harvested from embryos (36th passage) on the 17th day of incubation (11 days following yolk sac inoculation) was pooled, ground in a Ten Broeck tissue grinder, and frozen at –20 C until use. For vaccine preparation, the brain material was thawed and diluted to a 20 percent suspension with sterile distilled water. The pH was adjusted to 7.8 by the addition of M/1 Na_2HPO_4. The suspension was then centrifuged for 20 minutes at a relative centrifugal force of approximately 1000 x gravity. An aliquot of the supernate was removed, diluted 1:1 with sterile distilled water, and adjusted to pH 7.8. This aliquot was titrated in eggs prior to and following incubation at 37 C for 2 hours. Chilled 0.1 percent BPL* was added to an equal amount of supernatant fluid to give final concentrations of 10 percent brain supernate and 0.05 percent BPL. After thorough mixing, the suspension was incubated in a 37 C water bath for 2 hours. The pH was readjusted to 7.2 and the mixture stored at 4 C until use. Virus treated with BPL was tested for inactivation by injecting 0.5 ml into the yolk sacs of 15 susceptible 6-day-embryos. Embryos were examined on the 18th day for evidence of infection.[1]

Aluminum hydroxide was added to one lot of vaccine at a level of 6.25 percent to serve as an adjuvant.

Experimental birds. All birds used in these trials were progeny of a privately-owned flock of White Leghorns known

* Betaprone: Testagar & Co., Inc.

to be fully susceptible to AE. The chicks were hatched and raised in isolation units in wire batteries. Uninoculated controls were kept in the batteries with the vaccinated birds.

Inoculations. Intramuscular injections of 0.1 to 1.0 ml were administered with a 20 gauge 2 inch needle. All inoculations were given in the thigh. In trial 1, two groups received two injections at a 10-day interval while all others were given a single inoculation. Half of the groups in trial 2 were inoculated once, the others received a second injection 5 weeks later.

Serology. Preinoculation blood samples were taken at random just prior to vaccination. All birds were tested at 5 weeks postvaccination (trials 1 and 2) and again at 10 weeks postvaccination (trial 2 only). Serum-neutralization (SN) tests were conducted and neutralization indices (NI) calculated as previously described.[1] Previous work had indicated that no bird with an NI of 1.1 or higher succumbed to intracerebral challenge of 10,000 EID_{50} of AE virus.[1] Therefore, birds with neutralization indices of 1.1 or greater were considered immune.

RESULTS

Vaccine titers. Virus used for trial 1 had a titer of $10^{4.1}$ EID_{50}/0.1 ml prior to inactivation. Incubation for 2 hours at 37 C did not alter the titer. No evidence of infection was observed in 15 embryos inoculated with 0.5 ml of BPL treated virus indicating that inactivation was complete. Vaccine for trial 2 contained $10^{4.6}$ EID_{50}/0.1 ml prior to inactivation. Egg inoculations with BPL treated virus again indicated noninfectivity.

Trial 1. This trial was conducted with 7 groups of ten 11-week-old chickens. Preinoculation neutralization indices of 10 birds resulted in a geometric mean titer (GMT) of 0.3 with a range of –0.1 to 0.8. Detailed data on dosage, inoculation, and immune responses are found in table 1. It is seen that one inoculation of 12,500 EID_{50} was completely ineffective either with or without the $Al_2(OH)_3$ adjuvant. Either a second injection or a tenfold increase in dosage stimulated an immune response in some birds (roughly 30%) though in no instance

was an NI of greater than 1.4 observed. Negative controls (group 7) had no positive titers. No postvaccination reactions were evident.

TABLE I

Serological responses following intramuscular inoculation of susceptible 11-week-old chickens with BPL (0.05%) inactivated AE virus. Trial 1.

	Dosage per bird (EID_{50})		Immune response 5 wks postvacc.*		
Group	0 day	10 days	Immune/tested	GMT	Range (NI)
I	12,500	—	0/10	.4	0 to .8
II	12,500	12,500	3/9	.7	0 to 1.3
III	12,500+A**	—	0/10	.3	-.1 to 1.0
IV	125,000	—	3/9	.7	.1 to 1.4
V	125,000	125,000	3/9	.8	0 to 1.4
VI	125,000+A	—	3/10	.7	0 to 1.3
VII	—	—	0/10	.3	0 to .8

* Immune = neutralization index (NI) of 1.1 or greater. GMT = geometric mean titer.
** A = inclusion of 6.25% $Al_2(OH)_3$ in vaccine as an adjuvant.

Trial 2. In order to further test the effects of dosage and multiple injections, a second trial was conducted using forty 3-week-old and forty 12-week-old chickens. The experimental plans were identical for the two groups. Ten preinoculation SN tests from each group were negative. Inoculation data and results from the 3-week-old and 12-week-old groups are found in tables 2 and 3 respectively. Again, no postvaccination reactions were observed during the experiment. Of particular note were the following observations: 1) two injections of 200,000 EID_{50} were necessary to stimulate positive serological responses in all or nearly all birds, 2) in all instances, the second administration of vaccine served both to increase the percentage of positive birds and to raise the GMT, and 3) generally, the serological response was higher at 10 weeks than at 5 weeks following a single vaccination of 3-week-old birds. The latter suggests that peak titers had not been attained in all birds at 5 weeks postvaccination. In the case of the 12-week-old birds, however, GMT levels dropped between 5 and 10 weeks after a single injection. No other significant differences between the two groups were observed.

TABLE II

Serological responses following intramuscular inoculation of susceptible 3-week-old chickens with BPL (0.05%) inactivated AE virus. Trial 2.

	Dosage per bird (EID_{50})		Immune response*					
			5 weeks postvacc.			10 weeks postvacc.		
Group	0 day	5 weeks	Immune/tested	GMT	Range (NI)	Immune/tested	GMT	Range (NI)
I	40,000	—	0/5	.1	−.3 to .8	2/5	.7	.2 to 1.3
II	40,000	40,000	1/5	.3	0 to 1.2	3/5	.8	−.4 to 1.7
III	200,000	—	2/5	1.0	.3 to 1.8	3/5	1.1	.6 to 1.8
IV	200,000	200,000	2/5	1.0	.6 to 1.4	5/5	2.1	1.1 to 2.8
V	400,000	—	3/5	1.0	.3 to 1.5	2/5	.8	.1 to 1.1
VI	400,000	400,000	3/5	1.1	.8 to 1.5	4/4	2.5	2.3 to 2.6
VII	—	—	0/10	.2	−.1 to .6	0/5	.1	−.1 to .4

* Immune = neutralization index (NI) of 1.1 or greater. GMT = geometric mean titer.

TABLE III

Serological responses following intramuscular inoculation of susceptible 12-week-old chickens with BPL (0.05%) inactivated AE virus. Trial 2.

	Dosage per bird (EID_{50})		Immune response*					
			5 weeks postvacc.			10 weeks postvacc.		
Group	0 day	5 weeks	Immune/tested	GMT	Range (NI)	Immune/tested	GMT	Range (NI)
I	40,000	—	0/5	.1	−.4 to .5	0/5	0	−.1 to .1
II	40,000	40,000	0/5	0	−.2 to .3	2/4	.9	.1 to 1.8
III	200,000	—	3/5	.9	.5 to 1.3	0/4	.1	.2 to .9
IV	200,000	200,000	2/5	.9	.5 to 1.4	4/5	1.6	.6 to 2.8
V	400,000	—	4/5	.9	−.5 to 1.5	1/5	.9	.4 to 1.9
VI	400,000	400,000	2/5	.8	.4 to 1.4	5/5	2.9	2.4 to 3.9
VII	—	—	0/10	.3	−.1 to .8	0/4	.2	0 to .6

* Immune = neutralization index (NI) of 1.1 or greater. GMT = geometric mean titer.

DISCUSSION

It is evident from these results that a BPL inactivated virus retains enough antigenicity to stimulate a good immune response providing dosage is adequate. Neutralization indices following two injections of 400,000 EID_{50} inactivated virus compare favorably to those observed following intramuscular or wing-web vaccination with live virus.[1,2]

The inclusion of aluminum hydroxide as an adjuvant did not serve to increase the immunity in the two small groups tested (trial 1). These results are contrary to those observed by Schaaf.[4] The dosages of virus in the vaccines including aluminum hydroxide in the trials reported here, however, were considerably lower than those used by Schaaf (12,500 to 125,000 EID_{50} versus 1,600,000 CLD_{50}). Further investigation is necessary to account for the difference in results.

No postvaccination reaction was observed in any of the birds inoculated with the killed vaccines. It would seem likely that the use of such a vaccine would have no detrimental effects in adult breeders other than those brought about by handling. An important role of a killed vaccine will be the protection of susceptibe breeders on premises where the use of a live virus vaccine in young birds is anticipated. In these circumstances, this technique would need be employed only at the initiation of a vaccination program.

SUMMARY

The immune responses following intramuscular administration of beta-propiolactone (BPL) inactivated avian encephalomyelitis (AE) virus were studied. Consistent and high responses were obtained when two doses at 5 week intervals of 200,000 to 400,000 EID_{50} were employed. Neutralization indices following such a schedule were generally between 2.0 and 3.0 .

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THE DISTRIBUTION OF AVIAN ENCEPHALOMYELITIS IN NORTH AMERICA AS INDICATED BY AN IMMUNITY TEST

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SINCE the first observation of avian encephalomyelitis (AE) in 1930 by Jones[7] in a New England flock, the disease has been repeatedly observed in widely scattered areas of the country.[1,5] By 1950 it was apparent that the disease had been observed throughout the United States.[4] It has also been diagnosed in Canada,[14] Australia,[6] and Great Britain.[10]

It has been stated in the past that the disease appears to exhibit a seasonal periodicity. A minor period of prevalence is noted in the fall, and a major period in the spring months.[8] In recent years, the advent of more off-season hatching has tended to reduce this periodicity. Currently, the disease is observed to strike at any time when hatching is being done, especially if a young supply flock is being used.

Since the disease produces no visible symptoms in the laying flock during the time of egg transmission, with the

inconsistent exception of a temporary drop in production, it is impossible to estimate the incidence of AE in the field. If hatching eggs are not being saved at the time of infection, or if the flock is an unmated, commercial-egg flock, no conclusive evidence of infection is available.

It is apparent that some simple test for specific antibodies to this disease would greatly assist in determining its distribution and incidence. Recently, a serum-neutralization test has been described[2] but the laborious task of blood sample collection and the field problems of handling these samples may reserve this test for the more exacting requirements of controlled research projects. Early studies on the propagation of the AE virus indicated that this virus could only be grown by intracerebral inoculation of chicks. More recently it has been demonstrated that the virus can be grown in chick embryos inoculated intraocularly,[9] and even by the simpler technique of yolk-sac inoculation.[15] The source of eggs determines the success experienced in consistent propagation of the virus by either of these methods. The titration of AE virus gave varying results depending on the source of eggs.[9] A relationship was noticed between the history of previous avian encephalomyelitis in chicks from certain flocks and a low titration end-point in the embryos from these flocks. However, the slow procedure of intraocular inoculation, the necessity to prepare dilutions and the somewhat confusing occurrence of " 'accidental' survivals and deaths" limits the application of this procedure.

It occurred to the writers that if a standard dosage of virus could be used by a simple route of inoculation to separate those flocks which have had experience with the disease from fully susceptible flocks, an extensive field survey could be conducted. It is the purpose of this report to describe such a testing procedure and to record the findings obtained by application of the test.

MATERIALS AND METHODS

The test is founded on the observation that embryos from only certain sources will support the growth of the epidemic

tremor virus when inoculated by the yolk-sac route. During the preliminary development of this test, three different virus strains of different isolation source were used. No preference was observed, and it was concluded that any AE virus sample, the uniform growth of which is previously established in eggs, is suitable for the test.

A large number of embryos were inoculated at 6 days of incubation. A satisfactory source of embryos was found. The embryos were capable of supporting growth as demonstrated by typical embryonic changes. These changes were inactivity, dwarfing, and muscular dystrophy. Under aseptic conditions, the whole embryos were harvested at 14 days and ground in a Waring Blendor, distributed in aliquot samples, and frozen at –20 C. A titration was subsequently conducted on a sample of each pool, and was found to be in the range of 10^{-5} to 10^{-6} ID_{50}. The remainder of each pool was then used for test purposes. Numerous repeated trials indicated that a suitable dilution for inoculation was approximately $10^{3.5}$ ID_{50} per embryo. For example, if a given pool had a titration of $10^{-5.5}$ ID_{50} then a dilution of 10^{-2} was used. The diluent used in the preparation of virus for all embryo inoculations was physiological saline containing 5,000 units of crystalline penicillin G procaine and .005 grams of dihydrostreptomycin sulfate per ml.

Fertile eggs from supply flocks were incubated for 5 to 6 days, candled, and inoculated by the yolk-sac route. At 16 days the embryos were candled, the dead embryos were discarded, and the live embryos were opened for examination. Dwarfing, muscular dystrophy of the leg muscles and complete inactivity were observed in embryos from susceptible (S) flocks. Normal embryos were observed in eggs from resistant (R) flocks. As experience was gained, embryos could be examined at 14 rather than 16 days.

From table 1 it will be noted that in S flocks, regardless of the number of embryos inoculated, all embryos showed lesions. In R flocks, the converse was not always true. It was not unusual in these flocks to find a small percentage of embryos susceptible on inoculation. At first, this was inter-

preted to mean that all of the birds in the flock had not experienced the infection and these hens were producing susceptible embryos on inoculation. To study this point, such a flock was studied extensively with the aid of daily trap nesting for a period of 4 months. It was determined that certain hens contributed eggs which were always resistant to inoculations, while others contributed eggs which were occasionally susceptible to inoculation. When eggs from the latter groups were studied in detail and inoculated with different dilutions of virus, it was found that variations of the amount of virus inoculated resulted in an increase or decrease in the number of susceptible embryos. With sufficient dilution ($10^{1.0}$ ID_{50} per

TABLE I

Typical results obtained on testing of flocks for avian encephalomyelitis susceptibility

Flock	Eggs set	Infertile	Dead embryos	Lesions observed	No lesions	Flock status
A	100	10	5	85	0	S*
B	18	2	1	15	0	S
C	100	10	5	5	80	R**
D	18	2	1	1	14	R
E	18	2	1	5	10	R

*Susceptible (no previous infection).
**Resistant (previously infected).

embryo) practically no embryos showed lesions. All embryos, inoculated with this same dilution, from a known S flock showed lesions. These observations indicated that the occasional embryos showing lesions were not from susceptible hens, but rather from hens which possessed a lower level of antibody than pen mates. While the appearance of these occasional susceptible embryos from an R flock could be virtually eliminated by using a greater dilution of virus, it was decided not to make this change as the greater amount of virus would serve as a safety factor where large numbers of tests were to be run on a "production line" basis. If by some error the virus used was too greatly diluted, left out, or a given series of embryos was missed in the inoculation procedure, it might go unnoticed and all embryos would be normal whether originating from S or

R flocks. However, when even from R flocks the occasional affected embryo would occur, a "built-in" control would be provided which would preclude the possibility of misinterpretation due to absence of virus. With continued use, increased confidence in the test was developed, and this "built-in" control removed the necessity of inoculating known susceptible eggs as a control with each group inoculated. To further study the possibility of lack of uniformity of infection in supply flocks a number of other steps were taken. Eggs from hens in an R flock which frequently produced eggs susceptible to the high concentration of virus used were hatched out, as were eggs from pen mates which were always resistant. Chicks were also hatched from known S flocks. At one day of age each chick in the three groups was intracerebrally inoculated with dilutions of virus. The results obtained were as follows:

Source of chicks	Dilution of virus producing symptoms in 50% of chicks
R hens	$10^{-3.24}$
Questionable R hens	$10^{-4.23}$
S hens	$10^{-5.63}$

These results, and the degree of uniformity in the component groups making up these figures, were considered as further evidence that the hens in the R groups producing eggs susceptible to inoculation were merely carrying a lower level of immunity than their pen mates. Application of the serum-neutralization test[2] also indicated that the hens contributing the questionable R eggs possessed titers intermediate in range between birds laying S and R eggs.

In order to make the test practical, it was considered important to establish how many eggs should be tested from each flock. While previous observations had indicated that the likelihood of partially infected flocks was not great, this would have to be taken into consideration. Various quantities of eggs ranging up to 100 eggs were obtained from flocks and pens within flocks. Regardless of the sampling or source of eggs, the results, almost without exception, fell into two categories as indicated in table 1. Flocks A and B produced eggs which were 100 percent susceptible on inoculation. Flocks

C and D represent the largest group which uniformly produced none or only a small percentage of susceptible embryos. In a very small percentage of tests run, a result of the nature as seen in flock E (table 1) was obtained. At first this was interpreted as evidence that partial flock infection could exist; however, retesting provided a probable explanation of this situation. Invariably the next test run on such a flock (approximately one month later) gave results as seen in flocks C and D. This was interpreted as meaning that at the time of the original test, the antibody level was still rising. By the time of the second test, the level of immunity was uniformly high. When a full scale testing program was embarked on, which included the retesting of S flocks, this conception was confirmed. Occasionally, the first test run on a previously known S flock after infection would produce such a result. In other words, the testing sequence at monthly intervals was as follows: S, S, S, questionable R, R, R, and R. As a result of these observations, it was decided that 15 to 18 eggs were adequate for routine testing purposes.

The study of individual pens on premises provided interesting results. At no time was a house found to be made up of pens of both R and S ratings for more than one test. Subsequent tests always showed uniformity of infection which was interpreted as indicative of the highly infectious nature of the disease. Houses of birds separated by some distance, but on the same premises, and containing birds reared apart from one another, occasionally gave dissimilar results, i.e., one flock was R and the other remained S for several months running. This indicated the necessity of testing each house and each age group on each premises. However, it was most unusual for birds to persist in an S status after introduction to a premises where an R flock was being held, even if housed in a different building.

The above preliminary testings on flocks from 18 states were correlated with the presence and absence of AE in chicks hatched from S and R flocks during the 1958-59 hatching season. Not a single case of AE was observed in chicks from R flocks, while the usual number of cases experienced in a

TABLE II

Results of tests for epidemic tremor susceptibility conducted on 2,162 flocks from 46 States and Provinces.

State or Province	AGE and STATUS OF FLOCKS*																							
	5R	5S	6R	6S	7R	7S	8R	8S	9R	9S	10R	10S	11R	11S	12R	12S	13-18R	13-18S	19 & Older R	19 & Older S	? Res	? S	Total R	Total S
Alabama	2		1		2	2									2		1						8	2
Alberta							3		2												4		9	0
Arkansas			1		1				1						1								2	1
British Columbia					1				1		2												5	0
California	1		7		9	1	1						1								1		20	1
Colorado			1	1	2	1	2	1															5	3
Florida	1	3					1		2												2		6	3
Georgia	12	2	5	3	5	1	6		4	1	4	1	3		2		3						44	8
Idaho						1																	0	1
Illinois	8	7	33	19	67	18	44	6	16	4	4				1		1				11	1	185	55
Indiana		6	18	7	17	5	5	3	3	2	1		1				3				1		49	23
Iowa	10	5	68	5	140	9	80	7	37	2	10	1	5	2			1		1		23	1	375	32
Kansas	3		10	2	12	4	6	4	4	2	3	2							1		9	2	48	16
Kentucky			1		2		1																4	0
Louisiana			2		2				1								1						6	0
Maine	2																						2	0
Manitoba																					15	7	15	7
Maryland		3	2	1	1	2															3		6	6
Massachusetts											2						1						3	0
Michigan	1	1	4	1	13	4	8	2	4	2	1	1					1		1		4	1	37	12
Minnesota	23	13	44	1	61	1	30	5	8	2	1				1		3				7	1	178	23
Mississippi			3								1		2				1						7	0
Missouri	5	1	21	13	11	3	6	1	23	5	1						2				1	4	70	27

Montana					2	1	2	2	1	3																			5	6
Nebraska	5		40	5	70	9	24	15	9		2	2	2		1		2						2		157	31
Nova Scotia		2																							0	2
New Jersey							4		2																6	0
New York							1			4	1						1								3	4
North Carolina			1	1	5												1						2		9	1
North Dakota	2		2				1																		5	0
Ohio	2	5	35	29	33	13	17	11	5	3	2		1				6						1	3	102	64
Oklahoma		1	6	5	2	1	1	3		1							1								10	11
Ontario		3	3		3		2			1	1	1	1		1								16	1	27	6
Oregon					1																				1	0
Pennsylvania	3	4	20	15	17	13	8	5	1																49	37
Quebec					1						2				3										6	0
Saskatchewan							1		2		1						2								6	0
South Carolina	1	1	8	2	1		1		1		1				4		2								19	3
South Dakota	2		2	1	12	8	18	4	8	4	3												14	3	59	20
Tennessee	1		1	1			5																		7	1
Texas		2	1	3	5	2	3	6				4			1	2	6	2	2				10	4	28	25
Utah	1	4	5	3	2	1	1	1		1					1		1								11	10
Virginia	2	1	3	1	14												3								22	2
Washington					1						3														4	0
West Virginia			1																						1	0
Wisconsin	1	3	14	2	22	14	19	4	2	5	1	1					2		1				5	2	67	31
Total	88	67	365	122	537	115	300	81	135	40	47	13	16	2	18	2	45	2	6	0			131	30	1,688	474
Percentage P	56.8		74.9		82.4		78.7		77.1		78.3		88.9		90.0		95.7		100				81.4		78.1	

* Number refers to age of flock in months when tested, "R" refers to resistant status, and "S" refers to susceptible status.

**Question mark refers to flocks where exact age was not provided at time of testing.

hatching season were seen in S flocks as well as untested flocks. All flocks that produced AE infected chicks previous to our first test, were found to be R. In the light of these findings, it was decided to embark on a complete testing procedure of all commercial, hatching supply flocks and foundation supply flocks under the writers' control.

RESULTS OF TESTING SUPPLY FLOCKS

Table 2 summarizes the findings of the *first* tests conducted on 2,162 supply flocks from 46 different states and provinces. These tests were conducted during the months of July through December, 1959. The age of flocks at the time of testing varied from 5 to 18 months. The results of subsequent retesting of the same supply flocks are not included in table 2 but are included in the summary in table 3. The results presented in table 2 indicate the widespread distribution of AE. In the 46 states and provinces tested, all but Idaho and Nova Scotia had R flocks. The small sampling in these two regions (1 and 2 flocks respectively) is suggestive that further sampling might demonstrate the presence of the disease there as well. The oldest flock found to be susceptible was 17 months of age.

Numerous variables are encompassed in the summary in table 2. These include distribution of flocks in two countries over thousands of square miles, age at time of testing, number tested in each age group, varieties of chickens tested, rearing and housing husbandry, and climatic differences. Despite these variables, an age relationship is indicated by the percentages of R flocks. Only 6 of 85 flocks (7 percent) older than 10 months of age were susceptible and only 2 of 52 flocks (4 percent) older than one year of age were susceptible. On the other hand, over 43 percent of flocks were susceptible at 5 months of age.

The retesting of S flocks at monthly intervals was carried out on 258 flocks. The results are summarized in table 3. These data provide an indication of the average rate of infection under field conditions. Of 258 flocks S at 5 months of age, 42 became R (16.3 percent) during the next month. This

percentage increased monthly and by 12 months of age 98.6 percent of the susceptible flocks had become resistant. This retesting of S flocks has demonstrated the reliability of the test as not a single variation was observed, i.e., at no time did a flock which became R subsequently produce an S rating on repeated retesting.

TABLE III

Results of re-testing of susceptible flocks at monthly intervals.

	Age in months at time of re-test								
	5	6	7	8	9	10	11	12	13
Number of flocks R	0	42	74	102	127	134	138	140	140
Number of flocks S	258	216	178	125	55	24	8	2	2
Total tested*	258	258	252	227	182	158	146	142	142
Percentage becoming R		16.3	29.4	44.9	69.8	84.8	94.5	98.6	98.6

*Total of flocks tested is not consistent and decreases with age of flocks as several flocks included in data have not reached 13 months of age. Percentages are calculated on results of flocks actually re-tested in each age group.

DISCUSSION

The above data demonstrate the widespread distribution of this disease. Fortunately, a high percentage of supply flocks contract the natural infection during the growing or early laying periods. Despite the asymptomatic nature of the disease, the flocks are solidly immune by the time their eggs are used for hatching purposes. The remaining susceptible flocks will produce chicks with the disease if the hens are infected during the period that hatching eggs are being used. The devastating disease in baby chicks, and the permanent ocular damage to some survivors,[11] can be avoided by hatching only from R flocks.

Immunization has been successful for the wing web procedure using a live virus vaccine,[12] or a killed vaccine.[13] The necessity of individual handling and inoculation of birds when either of these procedures is used, and the rather frequent severe effects of the former procedure, indicate that the mass methods of immunization under study[3] will meet with greater

acceptance. It is conceivable that a combination of a testing procedure as described in this report and a mass immunization program may constitute an efficient program for breeding stock. By this approach, all flocks could be tested early in their production period and only the susceptible flocks would then require vaccination. This would result in considerable savings of money and effort.

SUMMARY

1. A simple test for immunity of breeder flocks to avian encephalomyelitis is described. The test depends on the failure of embryos produced by immune hens to support the growth of avian encephalomyelitis virus.
2. The presence of the disease was demonstrated throughout Canada and the United States. Embryos from 2,162 flocks from 46 states and provinces in Canada were tested. There was an increasing percentage of resistant flocks from 56.8 percent at 5 months to 95.7 percent at 13 to 18 months.
3. The use of this test to select resistant breeder flocks is suggested as a means of avoiding the disease in their progeny.

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THE DIFFERENTIATION OF *DERMANYSSUS GALLINAE* FROM *ORNITHONYSSUS SYLVIARUM**

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ONE problem that confronts all poultry pathologists is that of distinguishing between the northern fowl mite, *Ornithonyssus sylviarum*, and the red mite, *Dermanyssus gallinae*. There is a superficial morphological similarity between the two mites. Both are active blood suckers and can cause extensive damage. In moderate to heavy infestations both species can be found on the host, although *D. gallinae* is usually not seen on the chicken during the day. Accurate identification is pivotal because the methods of control differ for each species. Characteristically *O. sylviarum* pursues its entire life cycle on the host, while *D. gallinae* resides primarily in cracks around the wooden nests and roosts infesting the host only when obtaining a blood meal. To eliminate northern

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† This work was done while the author was a student in the Department of Entomology, College of Agriculture, Cornell University, Ithaca, New York.

fowl mites, the birds themselves need treatment, while for red mites, the woodwork and fixtures are treated. They are both in genera of the family of parasitic mites, Dermanyssidae. Although there are keys[3] which enable one to identify the various genera of parasitic mites, high quality preparations and a great deal of experience are required. The diagnostician requires a rapid, simple, yet accurate method of differentiation.

It is pertinent to review the methods in use currently. Reis and Nobrega[5] differentiated them on the basis that the chelicerae of the red mite are whiplike and work in a vertical, in and out fashion, while the chelicerae of the northern fowl mite possess minute shear-like structures at their ends that have a scissor-like action. One mounts the live mite in water, under a cover slip, and presses gently to cause extrusion of its chelicerae and observes their action under the microscope. Frequently, however, the chelicerae are not extruded leaving the issue in doubt.

Another method for distinguishing the two species is based on the life cycle of the mite. It is assumed that if mites are found on the bird they must be northern fowl mites. This is erroneous. Birds received at diagnostic clinics frequently are infested with red mites.

In this paper a simple, practical, highly accurate method is described that enables a differentiation based on the structure of the anal plate.

MATERIALS AND METHODS

The work reported here was based on the examination of more than 300 mites from 35 cases submitted to the five regional poultry diagnostic laboratories in New York State during the fall of 1957. These laboratories receive accessions from all of New York State and bordering areas of New Jersey, Connecticut, Pennsylvania, and Massachusetts. Thus the mite samples were taken not from an isolated population, but from a wide geographic range.

The parasites were collected and forwarded in formalin to Ithaca at monthly intervals. The mites were gently boiled

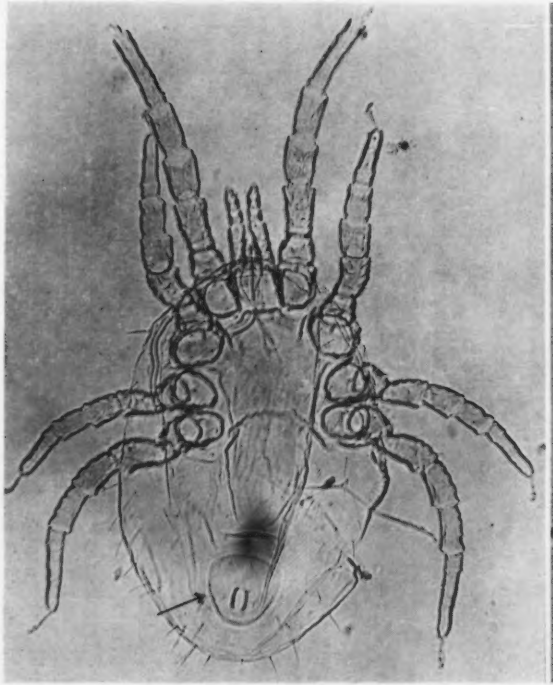

Figure 1. (left) Ventral view of female *Dermanyssus gallinae* with truncate or keystone shaped anal plate designated by arrow. X46

Figure 2. (right) Ventral view of female *Ornithonyssus sylviarum* with tear shaped anal plate designated by arrow. X46

in a 5 percent potassium hydroxide solution for 3 to 5 minutes to clear them of obscuring heme pigments. For a permanent preparation they were washed in two changes of water, and then mounted ventral side up in Hoyers solution[4], a modified Berlese media. If a temporary preparation was desired, they were mounted directly in water, ventral side up, under a cover slip.

The anal plate of a female northern fowl mite is discernable as a teardrop shaped structure at the terminus of the abdomen (figure 2). The anal plate of a female red mite is truncate or keystone in shape (figure 1). With a little experience observation of even a portion of the plate will suffice for differentiation. Generally, a magnification of 100 diameters is adequate to make the examinations. It should be stated that these structures were first studied in mites identified by the conventional keys. Living mites for this purpose were kindly furnished by Dr. R. K. Cole, Department of Poultry Husbandry, Cornell University.

DISCUSSION

This method, like almost any other diagnostic tool, has certain inherent limitations. Unlike lice, or helminth parasites which are generally species specific, mites may parasitize many different hosts. At least half a dozen species of mites usually foreign to chickens have been reported from them.[2] Fortunately, these occurrences are very infrequent. The tropical fowl mite, *Ornithonyssus bursa,* has occasionally been reported from the southern states. This method will not distinguish between the various species of *Dermanyssus* or *Ornithonyssus* but will serve to identify the genus. This is not a serious limitation inasmuch as the other and more unusual species are rarely encountered. The method of Reis and Nobrega suffers the same shortcoming, of course.

Male mites are described in the literature as possessing a fused genito-ventral plate.[1] The method herein described would not be applicable, therefore, to them. However, in all of the mites examined by the writer, the anal plates were as reproduced in figures 1 and 2 and described in the literature as

characteristic for female mites. Since our specimens were chosen at random, this would seem to indicate that the naturally occurring ratio of females to males at the time collections were made must be very high. Thus the rare presence of male mites would not interfere seriously with the selection of specimens for examination.

ACKNOWLEDGMENTS

Thanks are due to Drs. C. I. Angstrom, Saul Narotsky, M. C. Peckham, K. F. Hilbert, and Jean Hagan for collecting the specimens used in this study. The writer wishes to acknowledge the assistance and advice of Mr. John Mackiewicz of the Department of Entomology, New York State College of Agriculture, during the course of this study.

SUMMARY

A method is presented for the rapid differentiation between the northern fowl mite, *Ornithonyssus sylviarum*, and the red mite, *Dermanyssus gallinae*, on the basis of the anal plate structure. The anal plate in *Ornithonyssus* is teardrop in outline. *Dermanyssus* has an anal plate in the shape of a keystone. The limitations of this method for identification are discussed.

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NUTRITIONAL FACTORS INFLUENCING EXPERIMENTAL *CANDIDA ALBICANS* INFECTION IN CHICKENS. I. EFFECT OF VITAMIN A DEFICIENCY

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CANDIDA ALBICANS is a yeast-like organism frequently found on the mucous membranes and in skin lesions of most animals. The importance of this organism in producing disease and the frequency of its occurrence has been carefully reviewed by Chute and O'Meara.[5] Predisposing factors such as a lack of proper sanitation, and overcrowding have been considered important in the development of moniliasis.[8] Harris,[7] Reiches,[17] Keefer,[11] have emphasized the importance of human antibiotic therapy in favoring the development of candidiasis, possibly resulting from a disturbance in the microflora of the digestive tract.

The influence of purified, artificial diets high in glucose and antibiotics in predisposing chickens to moniliasis has been studied by Yacowitz *et al.*[24] Kenzy[12] had difficulty in reproducing thrush in normal chickens; however, when a diet

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Scientific Paper 1908, Project 1451.

[1] This is an extract from a thesis presented to the faculty of the Graduate School of Washington State University in June, 1959, in partial fulfillment of the requirements for the M.S. Degree in Veterinary Science.

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deficient in vitamin A was fed, moniliasis and death occurred. The object of this study was to investigate the influence of a vitamin A deficient diet on the production of moniliasis (thrush) in the chicken.

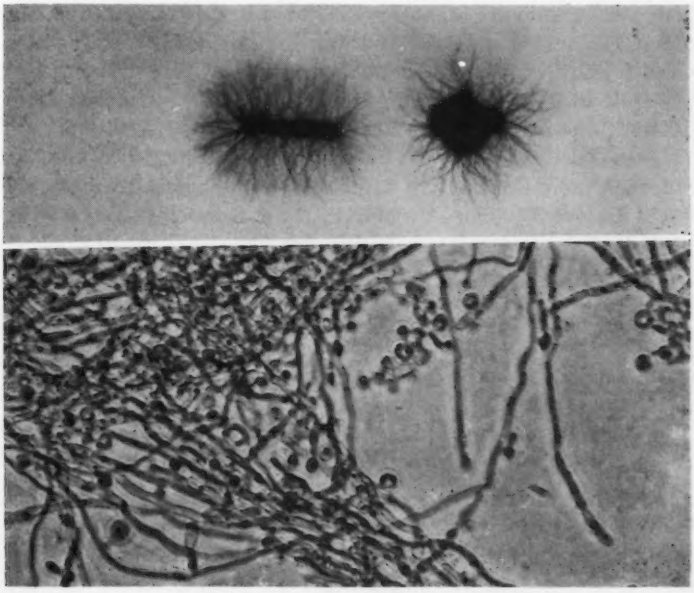

Fig. 1. (top) Spidery colonies produced by *Candida albicans* on E.M.B. Agar x 5

Fig. 2. (bottom) Chlamydospores on Liu's medium x 280 (phase contrast)

MATERIALS AND METHODS

A pure culture of *Candida albicans* originally isolated from a natural case of moniliasis in chickens was employed. The culture, which was found to be pathogenic for rabbits,* conformed to the classification of Skinner,[20] Martin *et al.*,[15] and Jungherr.[10] Typical, spidery colonies were produced (figure 1) on E.M.B. agar when incubated at 37 C under 10 percent CO_2 for one week.[22] A very slight pink pigmentation

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developed on Pagano-Levin medium after 3 to 4 days' incubation at room temperature.[16] The organisms grew luxuriantly on Sabouraud's agar at 37 C or room temperature; colonies were moist, raised and creamy-white. There was no surface growth in Sabouraud's broth. Young cultures consisted of oval budding cells, 3.5 to 5.5μ in diameter, while older cultures exhibited septate hyphae and blastospores. Occasionally, thick walled chlamydospores were observed at the ends of hyphae. Chlamydospore formation occurred after 16 to 18 hours incubation at 37 C on Liu's synthetic medium (figure 2). Three-week-old unsexed New Hampshire chicks, obtained from the Poultry Science Department of Washington State University, were divided into the following six groups:

Group A—8 chickens fed ration 1 (without vitamin A) exposed once.
Group AA—8 chickens fed ration 1 (without vitamin A) exposed twice.
Group B—7 chickens fed ration 1 (with vitamin A) exposed once.
Group BB—7 chickens fed ration 1 (with vitamin A) exposed twice.
Group C—14 chickens fed ration 1 (without vitamin A) not exposed.
Group D—14 chickens fed ration 1 (with vitamin A) not exposed.

Basic ration for all chickens was the same except that no vitamin A was added to the feed of groups A, AA, and C. The formula of this ration is given below.

RATION 1

Ground wheat	60.0 percent
Soybean oil meal (44 percent proteins)	35.0 percent
Bonemeal	3.0 percent
Limestone	1.0 percent
$MnSO_4$	0.0125 percent
NaCl	0.5 percent
Riboflavin	0.0004 percent
Vitamin D	150.0 I.C.U. per pound
Vitamin A	2000.0 I.U. per pound

Birds of Groups C and D which served as unexposed controls, were housed in a separate building and cared for by another attendant.

The inoculum used in the experimental work consisted of a 48 hour culture of *C. albicans* grown on Sabouraud's agar

and incubated at 37 C. The cells were harvested by emulsifying the growth in 0.85 percent saline solution and standardized to contain 145 million cells per ml by the direct count[1] and pour plate method.[1]

The inoculum of 145 million cells, was given by pipette directly into the crop when signs of vitamin deficiency were observed in chickens on a vitamin deficient ration.

Fecal counts were made twice a week on all groups. A large piece of stiff paper equal in size to the dropping pan was placed under the wire floor in each cage. After one hour each paper was removed and the droppings from each group were collected separately in sterile petri dishes and thoroughly mixed with a sterile spatula. Two grams of fecal material were taken from each specimen and emulsified in a Waring Blendor for 2 minutes with sufficient sterile distilled water* to give a 10^{-2} dilution of the feces. Ten-fold dilutions of the fecal emulsion were made through 10^{-6}. One ml of each dilution was then pipetted into a sterile petri plate and mixed with glucose tartaric acid agar** and incubated at 37 C for 48 hours. At the end of the incubation period, the number of colonies on each plate was counted under the Quebec colony counter. Identification of *Candida albicans* colonies was facilitated on this medium because of their typical gross appearance. The colonies were creamy white, round, convex and thin toward their periphery. The edges were fuzzy owing to radiating mycelia. The acidity of the medium helped to reduce the number of bacterial contaminants which might have been mistaken for Candida. The colonies were also examined under the low power objective for the presence of mycelia and blastospores. Smears made from colonies, picked at random, were stained either with methylene blue or rose bengal.

Twenty-seven days after the first exposure, the chickens

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*Distilled water was preferred to saline solution because it was observed from total counts on pour plate that the former has less deleterious effects on the yeast cells after emulsification and storage.

**Glucose tartaric acid medium which contains 5% glucose, 0.5% tartaric acid and 1.7% agar, was recommended by Dr. Charles Drake of the Department of Bacteriology and Public Health, Washington State University. This medium was found to be very useful in inhibiting all organisms except yeast and fungi because of its high sugar content and low pH.

of groups AA and BB were given another inoculation *per os* of a similar number of *Candida albicans cells* (145 million). Fecal counts were continued until the surviving birds were sacrificed on the 80th day after the first exposure.

On autopsy, the gastrointestinal tract was examined for gross lesions. Scrapings from apparently affected portions were examined under phase contrast microscope for the presence of mycelial structures by mounting in 10 percent potassium hydroxide or lactophenol cotton blue (Amman's medium)[20] Similar scrapings were also cultured for isolation of the yeast. In addition, the brain tissues were examined by wet mount treated with 5 percent potassium hydroxide and also cultured on Sabouraud's medium and glucose tartaric acid agar. Selected tissues were sectioned, stained with hemotoxylin-eosin[2] and Hotchkiss-McManus stains[13] and examined for the presence of yeast mycelia.

Pathogenicity of the cultures isolated from brains, and identified as *Candida albicans* was determined by animal inoculations. Three mice each were injected intraperitoneally with a mixture of 4×10^9 yeast cells and 2 mg of aureomycin.[19] One mouse injected with only the yeast, served as a control. In this manner, four mice were employed for each isolated strain.

Also, one rabbit was inoculated intravenously with 4×10^{10} cells of each of the isolates. These experimental animals were sacrificed 8 weeks after inoculation and examined for gross lesions and evidence of the presence of yeast in suspected organs. Selected tissues were sectioned and stained with Hotchkiss-McManus stain. Scrapings from affected organs were cultured on Sabouraud's and glucose tartaric acid media.

RESULTS

Prior to exposure with *Candida albicans*, clinical signs of avitaminosis A appeared in 63.3 percent of the chickens on a vitamin A deficient diet. They showed unthriftiness, an ocular exudate, lusterless feathers, dull shanks and retarded growth. These signs were not present in those fed a ration containing vitamin A.

Two chickens of Group A became partially blind owing to damaged corneas following excessive accumulation of exudate in the eyes. One chicken of Group AA showed swelling of the head from filling of the sinuses with a cheesy material. On autopsy, lesions of avitaminosis A were generally confined to the upper digestive tract and kidneys. They consisted of pustule-like lesions on the posterior surface of the pharynx and mucosa of the upper esophagus. Kidneys were enlarged owing to an accumulation of urates. In a few birds, deposits of urates were also observed on the intestines and lungs. It was found that the signs of vitamin A deficiency were more pronounced in those chickens which developed moniliasis (table 1).

TABLE I

Observations on the occurrence of avitaminosis A and moniliasis

		Number of chickens showing:					Mycelium[4] demonstrated in:	
Group	Wt.[1] (gm)	Sickness	Death	Clinical avitaminosis A	Nervous signs	Lesions of moniliasis	GI tract	Brain
A(—)[2]	1327	8/8[3]	6/8	7/8	5/8	7/8	6/8	4/8
AA(—)	1377	8/8	4/8	6/8	6/8	3/8	6/8	5/8
B(+)[5]	2262	1/7	0/7	0/7	0/7	1/7	1/7	0/7
BB(+)	2464	1/7	2/7[6]	0/7	0/7	0/7	0/7	0/7
C(—)	1820	1/14	0/14	6/14	1/14	1/14	1/14	1/14
D(+)	2268	0/14	0/14	0/14	0/14	0/14	0/14	0/14

[1] = Average weight of chickens 10 weeks after initiation of study.
(—)[2] = Ration 1 (without vitamin A).
[3] = Ratio of positive observations to the total number observed.
[4] = *C. albicans* demonstrated by culture on Sabouraud's medium.
(+)[5] = Ration 1 (with vitamin A).
[6] = One of these chickens had a large adenoma of the kidney which occupied most of the abdominal cavity; the other exhibited severe enteritis.

Clinical manifestations of illness became obvious 37 days after the first exposure when one chicken of group AA exhibited a lack of coordination, leg weakness and inappetence. No yeast could be cultured from the feces of this chicken. Similar signs gradually appeared in most of the other chickens in groups A and AA. Some showed gasping and difficult breathing accompanied by a slight rattle. This respiratory difficulty was found on autopsy to be due to necrotic tissue

masses in the trachea which obstructed free passage of air.

Most of the chickens in group A (62.5 percent), and in group AA (75.0 percent) showed nervous signs, such as, "star-gazing" (figure 3), shivering of head and neck, evidence of blindness, backing up against the cage walls and walking in a wobbly fashion. They acquired a typical standing posture with the crop held high and neck retracted in a pigeon-like fashion. Deaths associated with moniliasis were first noted on the 39th day after the initial exposure. Seventy-five percent of the chickens in group A and 50 percent of those in group AA died before the experiment was concluded on the 80th day after the initial exposure. Surviving birds in these groups were seriously affected and probably would have died if they had not been sacrificed.

Mycelial elements were demonstrated in brain tissues of 50 percent of group A chickens and in 62 percent of group AA. *Candida albicans* was recovered from the crops of 75 percent of the chickens of group A and AA. None of the chickens from group B died (table 1); however, one of them showed evidence of moniliasis and was sacrificed. Lesions of moniliasis were observed in the crop from which *Candida albicans* was recovered. Two chickens of group BB died. One had a large adenoma of the kidney and the other showed extensive hemorrhagic enteritis. No yeast or intestinal parasite could be demonstrated in these birds (table 1).

One out of 14 chickens in group C developed a typical, pigeon-like posture and slight signs of a nervous disorder as manifested by shivering and shaking the head. When this chicken was killed at the termination of the experiment, *Candida albicans* was cultured from the crop and brain and mycelial elements were demonstrated in these tissues in wet mount. The proventriculus of this bird was greatly enlarged and swollen; however, no lesions of moniliasis were observed in this tissue nor could the yeast be cultured. Feces from this unexposed group remained negative for Candida cells.

No bird of group D showed any signs of moniliasis, nor did they shed any yeast cells in their feces. They grew into healthy, apparently normal chickens. Scrapings from the

Fig. 3. (top) Chicken showing "star-gazing" attitude. Chicken A does not show signs of a brain infection even though both birds were fed the same vitamin A deficient ration and exposed to *C. albicans.*

Fig. 4. (middle) Lesions of moniliasis in the upper digestive tract.

Fig. 5. (bottom) Mycelial elements in the crop mucosa (Hotchkiss-McManus stain), x 200.

upper digestive tract were negative for mycelia on both cultures and wet mount examination.

Lesions of moniliasis in the affected chickens were again confined to the upper digestive tract, with most characteristic changes occurring on the mucosa of the crop (figure 4). Macroscopically, they appeared as whitish, irregularly raised, diphtheritic areas which were easily removed from the thickened mucosa. The mouth and esophagus also showed similar lesions in the severely affected chickens. In some chickens, the wall of the proventriculus was diffusely swollen and appeared glossy on the serosal surface. The mucosa showed ulcerations but no diphtheritic areas as in the case of mouth and crop lesions. The keratinized mucosal layer of the gizzard lost its glossiness and appeared opaque and greyish-white in certain localized areas. Ulcerative patches were also present in the mucosa of some of the gizzards.

No yeast cells were observed in the fecal cultures of exposed chickens until the 11th day after exposure (figures 6, 7). Then the number of colonies in pour plates remained practically constant until the 25th day.

Chickens of groups AA and BB were re-exposed on the 27th day after the initial exposure. On the second day following the re-exposure, a sudden increase in the number of yeast cells shed in the feces by these two groups was observed. There was also a slight rise in the number of yeast cells shed by the chickens of group A. No Candida cells were isolated from the feces of any of the four exposed groups after the 58th day following exposure. The number of yeast cells shed in the feces of chickens of group B remained low and showed little variation (figure 7).

The unexposed chickens of groups C and D, did not shed any yeast cells in their feces. Fecal cultures of individual birds at the time of sacrifice failed to show the presence of Candida cells.

Histological examination of tissue sections stained by the Hotchkiss-McManus method revealed the presence of mycelial elements in the superficial layer of the crop mucosa (figure 5). No mycelia could be detected in proventriculus,

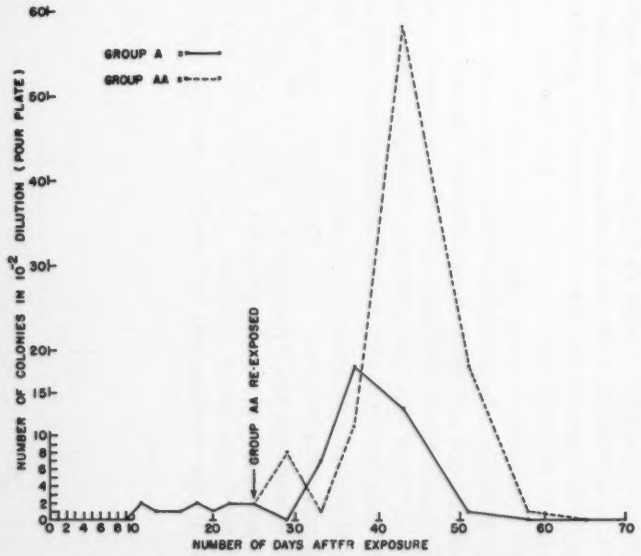

Fig. 6. Fecal counts of groups A and AA.

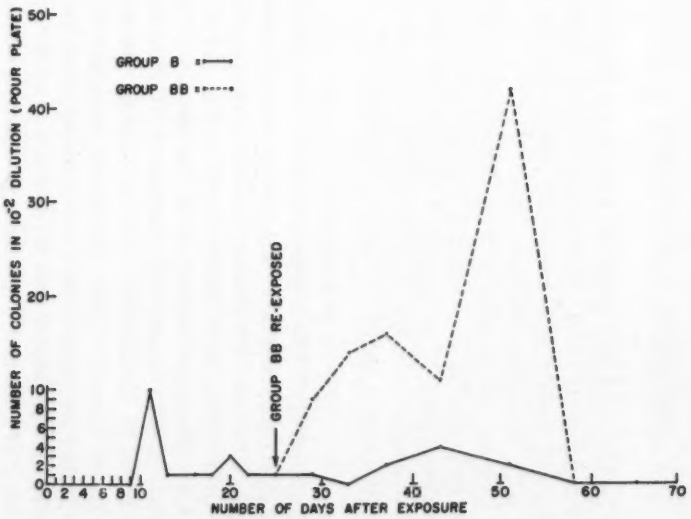

Fig. 7. Fecal counts of groups B and BB.

gizzard or brain. It was observed that the mycelium did not stain conspicuously with hemotoxylin-eosin; however, the fungi stained deeply and stood out clearly in the sections treated with Hotchkiss-McManus stain.

C. albicans was reisolated from kidneys of 50 percent of the surviving rabbits necropsied 8 weeks after inoculation to check pathogenicity of isolates. Although lesions were present in the kidneys, no mycelia were observed in sections. None of the inoculated mice showed evidence of infection.

DISCUSSION

Only one out of 14 chickens (table 1) on a balanced diet (groups B, BB) developed a mild clinical case of experimental moniliasis. However, the disease became severe in 62 percent of the chickens kept on a diet deficient in vitamin A, (groups A and AA). Adequate vitamin A in the diet was found to be important in maintaining the resistance of chickens which were exposed to *C. albicans*. This confirmed the earlier observations of Kenzy.[12] Other suggested predisposing factors such as improper sanitation, overcrowding, and respiratory diseases did not influence this study.

In view of Conant's[6] observations that meningitis in man is a serious sequela of moniliasis during intensive antibiotic therapy and avitaminosis, it was interesting to find that nervous signs were present in over 60 percent of the chickens on a vitamin A deficient diet (groups A, AA, table 1). Avitaminosis-A produces nervous disorders a result of pressure of slowly growing osseous cavities[21,23] on the normally growing brain, spinal cord and nerve roots. Some of the nervous signs may have been due to infection, since *C. albicans* was isolated by cultures from the brain of these chickens and mycelial elements were demonstrated in wet mounts. Only one of the 14 unexposed chickens of group C fed a diet deficient in vitamin A developed nervous signs and was found positive for *C. albicans* in cultures and wet mounts (table 1). Since none of the exposed chickens on the complete ration (groups B, BB and D) developed brain infection, importance of vitamin A preventing the involvement of central nervous system is

evident. Possibly the nervous signs observed by Blaxland *et al.*[4] in poults with moniliasis were due to *C. albicans* although no reference was made to cultural studies of the brain.

Although the yeast infection was demonstrated by wet mounts and by cultural examinations of brains from chickens showing nervous signs, the mycelial elements could not be demonstrated by histological studies of brain sections. Possibly the invasion of the brain tissue was quite limited and a more detailed examination would be necessary.

Sebrell, *et al.*[18] reported that a deficiency of vitamin A in chickens was observed to retard the growth. Chickens from groups B, BB, and D fed a complete ration weighed an average of 800 grams more than those from groups A, AA and C which were fed a ration without vitamin A (table 1). Moniliasis helped to retard the growth further, since the average weight of the uninfected chickens of group C was 500 grams more than that of the exposed chickens of groups A and AA. Moniliasis also aggravated the avitaminosis A, since 80 percent of the chickens in exposed groups A and AA exhibited clinical avitaminosis A as compared with 42.8 percent of unexposed chickens in group C on this same diet, (table 1).

Disappearance of *C. albicans* from the feces of all the chickens (table 1), sixty to seventy days after exposure, might be attributed to the development of acquired resistance.

Candida albicans has been found as a commensal in the crops of turkeys and chickens, without causing frank clinical manifestations even though gross lesions may be present.[3,4,9,14] When an organism with a potential for pathogenicity is found occurring "naturally" in animals without causing a recognized disease, it becomes difficult to explain whether an increase in virulence of that organism, or a lowered resistance of the host is responsible for the establishment of disease.

ACKNOWLEDGMENTS

The cooperation of members of the Veterinary Pathology Department is greatly appreciated, particularly that of Doctors R. W. Leader and J. L. Palotay for photographing the

experimental animals and specimens and Mrs. M. G. Eastlick for supervision of tissue section preparation and staining. Dr. Leo Jensen, Department of Poultry Science, kindly formulated and made available the feeding rations employed.

SUMMARY

1. The experimental production of moniliasis in chickens fed a vitamin A deficient ration was investigated.

2. Lesions of moniliasis appeared in over 60 percent (10/16) of the monilia-exposed chickens on a vitamin A deficient ration and in only 7 percent (1/14) of those on a complete ration.

3. Nervous signs and a definite retardation of growth were observed in the infected chickens on the vitamin A deficient ration.

4. Signs of vitamin A deficiency were twice as frequent in the monilia-infected chickens as in the non-infected controls.

5. *C. albicans* appeared in the feces within 11 days after the first exposure. Two days after the second exposure, 27 days later, a three fold yeast cell increase was observed in plate counts of the feces.

6. All shedding of yeast cells appeared to cease 58 days after the initial exposure, although frank lesions of candidiasis were still present.

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THE CULTIVATION AND MODIFICATION OF THE AVIAN INFECTIOUS SYNOVITIS AGENT IN TISSUE CULTURE

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ATTEMPTS have failed to cultivate the organism causing avian infectious synovitis (AIS) in cell-free culture media.[4,5,6,12] Lecce *et al.*[5] reported on the propagation in tissue culture of an agent that caused a tendovaginitis with arthritis in chickens. The method employed was the roller-tube technique using embryonic chicken heart. The criterion of growth was cytopathogenic activity. No reports have been made of serial passage of the agent in tissue culture or the infectivity for chickens of the tissue-culture-propagated agent.

This paper presents a method for propagating the AIS agent in tissue culture using the Maitland technique and describes the characteristics of the agent grown under these conditions.

MATERIALS AND METHODS

Tissue Culture Media. The medium used was Simm-Sanders medium composed of 80 parts of Simm-Sanders salt solution, 1 part of a saturated solution of p-amino-benzoic acid, 1 part of a 5 mg percent solution of cysteine hydrochloride, and 20 parts of bovine serum ultrafiltrate.[9] About 0.1 gm of minced chicken embryo was suspended in 10 ml of the nutrient medium in a 20 x 150-mm test tube. Penicillin was added to a concentration of 100 units per ml of medium.

Inoculum. The AIS agent used was isolated in California on March 2, 1955, and designated strain C-1. The gross

and histopathologic lesions produced by this agent in chickens and turkeys were indistinguishable from those described by Wills,[12] Cover *et al.*,[4] Olson *et al.*,[6] and Sevoian *et al.*[8] The initial inoculum for serial passage consisted of 0.1 ml of ground yolk sac suspended in allantoic ammiotic fluids (aafl) of the sixth embryo passage, which had an embryo lethal dose 50 percent (ELD_{50}) titer of 8.1×10^5 per 0.2 ml. For subpassage, 1 ml of the supernatant fluid of the previous passage was used to inoculate the fresh culture.

The inoculum of the virulent agent used for challenge consisted of ground yolk sacs suspended in aafl of the seventh chicken-embryo passage of the C-1 strain. The challenge dose was 0.2 ml with an ELD_{50} titer of 2.8×10^4.

All inoculations of chickens with infected tissue cultures or egg-propagated suspensions were made intravenously.

Titration. Cultures were titrated by inoculating 0.2 ml of supernatant tissue-culture fluid into the yolk sac of 5 to 7-day-old embryonating chicken eggs. Tryptose broth was used in making serial dilutions. Titers (ELD_{50}) were calculated by the method of Reed and Muench.[7]

Test Birds. All test birds were obtained as day-old chicks from the University flock, which is under constant surveillance for disease. The flock has been free of the disease under investigation, and is not vaccinated for the usual diseases encountered in poultry. The chicks were housed and raised in an isolated house until used.

Fertile Eggs. All eggs used for preparing tissue culture were collected from unvaccinated chickens raised in isolation and free of disease.

RESULTS

The AIS agent was grown in tissue culture through 50 serial passages of Simm-Sanders medium containing bovine serum ultrafiltrate. The presence and quantitation of the agent in tissue culture were determined by its pathogenicity for chicken embryos and its pathogenicity for susceptible chickens or ability to immunize them. The unit of quantitation used was the ELD_{50} since chicken embryos were found to be more susceptible to infection than the chicken (table 1).

TABLE I

Comparative titration of egg-propagated AIS agent[1] in embryonating eggs and 6-week-old chickens

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Embryos[2]	10/* /10	8/ /8	9/ /9	8/ /8	9/ /9	4/ /10	1/ /10	$ELD_{50} = 8.13 \times 10^5$/ /0.2 ml
Chickens[3]	5/** /5	4/ /5	3/ /5	0/ /5	0/ /5	0/ /5	0/ /5	$ID_{50} = 1.0 \times 10^3$/ /0.2 ml

[1] Sixth egg passage
[2] 0.2 ml inoculated into yolk sac of 6-day-old embryos
[3] 0.2 ml inoculated intravenously
* Number died/ /number inoculated
** number positive lesions/ /number inoculated

The titers of early tissue-culture passages varied from 6.8×10^5 (second passage) to as low as 3.0 (12th passage). The decrease in titer was believed due to a suboptimal incubation period since a study of the relation between culture titer and incubation period (table 2) showed that titers increased when incubation period was decreased from 96 to 72 hours. Incubating the tissue culture for 72 hours between passages gave consistently higher titers, ranging between 10^5 and 10^6 from the 14th to the 34th passage (table 2).

TABLE II

Titration of tissue-culture-propagated AIS agent in chicken embryonating eggs

Passage No.	Incubation Period in Tissue Culture (hours)	ELD_{50} per 0.2 ml
1	72	——
2	96	6.8×10^5
3	72	4.2×10^5
4	96	3.2×10^4
5	72	6.8×10^4
6	96	3.9×10^3
7	96	2.2×10^3
8	96	4.5×10^1
9	72	2.2×10^3
10	96	7.5×10^1
11	72	3.2×10^2
12	96	3.0
13	72	6.8×10^2
14–17	72	$>1.0 \times 10^4$
18	72	1.0×10^3
19–34	72	1.0×10^5 to 7.9×10^5
35	72	2.6×10^4
36	72	6.3×10^3
45	72	1.5×10^4
50	72	1.8×10^4

$>$ Greater than

The tissue-culture-propagated agent, inoculated intravenously into susceptible chickens, remained pathogenic through the ninth serial passage. Subsequent tissue culture passages produced no signs of disease, and challenge of asymptomatic birds with a virulent culture revealed that they were completely immune or so protected that no systemic reaction occurred and only mild joint lesions were observed at necropsy (tables 3 and 4). In contrast to the loss of pathogenicity for chickens, the tissue-culture-propagated agent maintained its pathogenicity for embryonating eggs throughout the study.

TABLE III

The response of chickens to serial passages of tissue-culture-propagated AIS agent

Passage No.	Age of Bird (wks)	Dose of TC Inoculum (ELD_{50})	Lesions	Response to Inoculum Incubation Period (days)	Course (days)
1	6	——	+	4	7*
2	6	3.4×10^6	+	7	11 (died)
3	3	1.0×10^6	+	6	11*
	3	5.2×10^6	+	6	11 (died)
4	4	1.6×10^5	+	3	7*
	4	1.3×10^6	+	9	14*
5	4	3.4×10^5	+	11	17 (died)
	4	3.4×10^6	+	6	11*
6	5	1.9×10^4	—	—	30
	5	1.8×10^5	+	—	28
7	5	1.1×10^4	+	11	35*
	5	3.3×10^4	+	18	35*
	5	7.8×10^4	+	13	21*
8	6	4.5×10^2	—	—	31
	6	2.0×10^3	—	—	31
9	6	2.2×10^4	+	18	21*
	6	1.1×10^5	+	10	21*
10	6	1.1×10^3	—	—	24
	6	1.1×10^4	—	—	23
11	6	3.2×10^3	—	—	41
	6	3.2×10^4	—	—	41
12	7	4.7×10^1	—	—	37
	7	4.7×10^2	—	—	37
13	7	1.0×10^4	—	—	34

* Extreme depression, emaciation and joint involvement at the time of necropsy.

During the first nine serial passages, the incubation period and course of disease in chickens increased with each passage. The increase was believed to be due, in part, to the decreased concentration of the agent. Inoculation of 1.9×10^4 ELD_{50} of passage six failed to produce disease, whereas 1.9×10^5 ELD_{50} of the same passage and 1.1×10^4 ELD_{50} of passage seven produced clinical infection. Thus, the minimum infective dose appeared to be between 1.1×10^4 and 2.0×10^4 ELD_{50}, considerably higher concentrations than observed for the initial egg-propagated agent, which produced the disease in four of five birds inoculated with 8.1×10^3 ELD_{50} and three of five birds receiving 8.1×10^2 ELD_{50} (table 1).

The exact passage in which the culture became apathogenic for chickens is unknown, for the asymptomatic birds were not challenged until the 14th passage, and it is probable that the doses of the 10th to 13th passages (possibly excepting the 11th) were less than infective. The instability of the organism under freezing and storage in tissue-culture medium prevented more accurate determination of the first apathogenic passage.

Table 4 shows the response to challenge, with a virulent culture, of birds previously inoculated with the tissue-culture-propagated AIS agent. Eleven passages (14, 16, 20, 21, 24, 25, 28, 30, 31, 33, 34) provided complete protection against any signs of disease, and three passages (18, 19, 26) provided complete protection only at the higher dose. Five passages (22, 23, 32, 35, 36) provided protection against systemic infection and death, but failed to prevent localization of the agent in the synovial tissues. Even in these cases the chickens appeared clinically normal; only at necropsy were minor localized lesions observed. All control birds were readily infected upon challenge, and either died or were necropsied in a weakened, emaciated state by the 3rd week.

Subculture of the Tissue-Culture-Propagated Agent in Eggs. The lesions observed in embryos that died or were infected with the AIS agent have been described previously.[3,11] Suspensions of yolk-sac and aafl collected from the first sub-

TABLE IV

The response to challenge of asymptomatic chickens previously inoculated with the tissue-culture-propagated AIS agent

TC Passage No.	Age of Bird (wks)	Dose of TC Inoculum (ELD_{50})	Response to TC Inoculum	Time in days between TC Inoc. and Challenge	Challenge* Response**	Days After Challenge
14	7	$>5.5 \times 10^5$	—	50	—	62
16	8	$>5.5 \times 10^5$	—	44	—	62
18	9	5.0×10^3	—	38	+	62
	9	4.5×10^4	—	38	—	62
19	9	1.0×10^5	—	35	+	62
	9	5.0×10^5	—	35	—	62
	9	5.0×10^6	—	35	—	62
20	10	3.3×10^5	—	32	—	62
	10	1.0×10^7	—	32	—	62
21	10	1.6×10^6	—	29	—	62
	10	7.9×10^6	—	29	—	62
22	11	1.6×10^7	—	26	+	62
23	11	1.7×10^7	—	33	+	53
24	12	9.2×10^6	—	30	—	53
25	4	4.5×10^6	—	39	—	36
26	4	3.9×10^6	—	36	+	36
	4	4.0×10^7	—	36	—	15
28	5	1.8×10^6	—	30	—	15
30	4	3.8×10^6	—	24	—	15
31	5	1.3×10^7	—	21	—	15
32	5	1.8×10^5	—	21	+	31
33	6	1.3×10^6	—	32	—	28
	6	1.3×10^7	—	32	—	28
34	6	1.2×10^6	—	29	—	28
35	4	6.4×10^5	—	26	+	28
36	4	1.6×10^5	—	23	+	28

* All birds challenged intravenously with 2.8×10^4 ELD_{50} (7th embryo passage).

** Exudate in one or more joints was considered a positive response. Susceptible chickens of the same age were used as controls in each instance. The controls either died or were severely depressed and diseased when necropsied.

culture tissue-culture-infected eggs were inoculated intravenously in susceptible chickens in 0.2-ml volumes (table 5). Egg material from the first 12 and the 14th and 17th tissue-culture passages were found to be infective. It is interesting that disease was produced by passages 8, 10, 11, and 12, in which the tissue culture contained less than an infective dose (1.1 x 10^4 ELD_{50}). It is also seen that early apathogenic tissue-culture passages (14 and 17) were pathogenic after one subculture in embryos.

TABLE V

The susceptibility of chickens to yolk-sac suspensions of the first embryo subculture of the AIS tissue-culture-propagated agent

** Egg Material Representing Tissue Culture Passage No.	Age of Bird (weeks)	Lesions	Response to Inoculum Incubation Period (days)	Course (days)
2	4	+	3	7 *
3	4	+	5	12 died
4	5	+	6	12 died
5	5	+	8	14 *
6	6	+	5	16 *
7	6	+	5	18 *
8	6	+	5	15 died
9	7	+	7	34 *
10	7	+	13	34 *
11	7	+	13	34 *
12	9	+	14	22 died
14	9	+	26	33 died
17	3	+	16	20 died

** Ground yolk-sac suspended in allantoic-amniotic fluids
* Extreme depression, emaciation and joint involvement at time of necropsy.

A single subculture in embryonating eggs of the 13th, 15th, 18th, and all subsequent tissue-culture passages was found to be apathogenic for chickens (table 7). An exception was the 32nd passage: one of four birds inoculated with subculture of that passage had minor wing-joint lesions on the 29th day. Though a single passage in eggs appeared to have little effect on the pathogenicity of the tissue-culture-modified agent, a second subculture in embryos resulted in nearly complete reversion to pathogenicity (table 6).

Birds that failed to develop disease following inoculation with egg-passaged material were challenged. The results were

necropsied.

similar to those obtained with birds that received cultures of apathogenic tissue-culture material (table 7). Of 14 birds challenged—each representing a separate passage—seven were completely refractory (passages 13, 15, 19, 21, 23, 24, 26) and seven had localized lesions on necropsy (passages 18, 20, 25, 27, 28, 29, 33). Two of three birds that received the same culture (passage 32) were refractory, and the third developed joint lesions.

TABLE VI

Comparative concentration and pathogenicity of the AIS agent in tissue culture and embryonating eggs

Source of Agent	ELD_{50} titer per 0.2 ml	Pathogenicity for Chickens (I.V. inoc.)
Tissue Culture Pass #32	1.8×10^5	0/4 *
Egg subculture #1 of T.C. pass 32	1.62×10^7	1/4 **
Egg subculture #2 of T.C. pass 32	5.58×10^6	4/4
	5.58×10^3	3/3

* No. infected/No. inoculated

** Small amount of exudate observed in one wing joint. Bird otherwise normal.

Stability and Storage of Tissue-Culture-Propagated AIS Agent. Tissue-culture suspensions of the AIS agent were stored at 0 C and –76 C. Storage for 5 days rendered the cultures innocuous for embryonating eggs or susceptible chickens and for growth in fresh tissue-culture medium (table 8). Adding 20 percent peptone to the media failed to protect the agent in the frozen state. Adding sucrose to a final concentration of 0.4 percent, however, protected the tissue-culture-propagated agent against instability during freezing and storage for at least 66 days (table 8). The agent propagated in eggs was stable when frozen in a suspension of yolk sac and aafl. In infected liver, kidney, spleen, or joint exudate, the agent maintained viability for 774 days at –76 C, the longest period tested.[10]

DISCUSSION

The ability to grow the agent causing AIS in chickens and turkeys in tissue culture provides another means of

studying the nature of this organism. Unfortunately, the technique used does not lend itself to quantitative determinations. Such determinations can be made in either the chicken or chicken embryo, though the latter was more sensitive to infection.

Simm-Sanders medium and the Maitland technique of tissue culture were chosen because of simplicity. Titers thus obtained were generally between 10^5 and 10^6 ELD_{50}. Incubation time was important in obtaining satisfactory titers. A growth curve was not established for the agent in tissue culture, but 72 hours of incubation were more satisfactory than 96 hours, suggesting that prolonged incubation may be detrimental to survival of the organisms. A rapid loss of viability was demonstrated following freezing of tissue-culture suspensions, in contrast to a greater stablity of the egg-propagated agent stored in yolk and aafl. The difference in stability

TABLE VII

The response to challenge of asymptomatic chickens previously inoculated with yolk-sac suspensions of the first embryo subculture of the AIS tissue-culture-propagated agent

Egg Material Representing Tissue-Culture Passage No.	Age of Bird (weeks)	Response to T.C. Inoculum	Time between T.C. inoculum and challenge (days)	Challenge* Response**	Days after Challenge
13	9	—	34	—	62
15	9	—	34	—	62
18	3	—	52	+	17
19	3	—	52	—	28
20	3	—	52	+	28
21	3	—	52	—	28
23	3	—	52	—	28
24	3	—	52	—	28
25	5	—	38	+	28
26	5	—	38	—	28
27	5	—	38	+	28
28	5	—	38	+	6
29	4	—	38	+	28
32	6	—	29	—	28
	6	—	29	—	28
	6	—	29	+	28
	6	+			
33	4	—	23	+	28

* All birds challenged intravenously with 2.8×10^4 ELD_{50} (7th embryo passage).
** Exudate in one or more joints was considered a positive response. Susceptible chickens of the same age were used as controls in each instance. Controls either died or were severely depressed and diseased when necropsied.

is probably not related to the method of growth, but is a function of the medium in which the agent is suspended, for sucrose added to the tissue-culture suspension was effective in maintaining viability following freezing and storage.

The virulence for chickens of the AIS agent gradually decreased during the first nine serial passages in tissue culture, and subsequent passages were relatively or completely apathogenic. Attenuation of the culture was apparent, though the loss in virulence was believed to be due in part to a de-

TABLE VIII

The effects of storage on the tissue-culture-propagated AIS agent following certain treatments

T.C. Passage Number	ELD_{50} titer	Pre-storage treatment	Temp. C	Time Stored (days)	ELD_{50} titer
P-2, series I	1.6×10^4	None	0	51	innocuous
subculture 1					innocuous
subculture 2					innocuous
P-8, series I	2.6×10^4	None	0	10	innocuous
P-9, series I	1.5×10^5	None	0	23	5.8×10^2
subculture 1					innocuous
P-4, series II	4.0×10^5	None	−76	5	innocuous
subculture 1					innocuous
subculture 2					innocuous
P-7, series IV	2.0×10^4	20% peptone	−76	14	innocuous
subculture 1					innocuous
subculture 2					innocuous
P-19, series V	1.0×10^5	0.4% sucrose	−76	66	$> 1.0 \times 10^4$
subculture 7					2.4×10^5
P-24, series V	1.8×10^5	0.4% sucrose	−76	3	———
subculture 1					3.1×10^1
subculture 6					1.0×10^5
subculture 9					1.7×10^5

creased concentration of the agent. This conclusion is based on the fact that, though titers decreased during the initial tissue-culture passages, the minimum infective dose to produce lesions or death increased over 90 percent by the seventh passage.

The modified culture retained its antigenic properties, as demonstrated by the development of immunity following intravenous inoculation. The dose or route of inoculation required for complete immunity is not discussed; a paper on this subject is in preparation. However, it should be noted

that all birds receiving an apathogenic culture were protected against challenge with a virulent AIS culture. Protection varied from complete resistance to resistance against death and systemic infection. Synovial lesions were less severe in partially protected birds, and fewer joints were involved than in control birds.

Apathogenicity of the culture was not a fixed characteristic. Up to the 17th passage level (excepting the 13th and 15th passages), apathogenic cultures reverted to a pathogenic state following a single subculture in embryos. This was in some cases due to the fact that higher titers are obtained in embryos than in tissue culture, so that subminimal infective dose levels could be raised to the infective level by passage in embryos. More important, however, is the fact that the chicken embryo favored pathogenicity, as demonstrated at the 32nd passage level, when the pathogenicity for chickens of the tissue-culture-propagated agent was reversed from apathogenic to pathogenic by two subcultures in embryos.

The results permit no more than speculation on the nature of the modification of the agent. Whether apathogenicity is a genetic or non-genetic variation is yet to be answered. Adaptation involving genetic variation would require mutation of the agent from a pathogen to a non-pathogen, with consequent selection of the nonpathogenic fraction. Nongenetic variation would involve a phenotypic accommodation, which could be reversed by passage in suitable media. Either case could be argued from the results presented; however, the authors favor the theory of genetic variation and the belief that the tissue-culture methods employed select for the apathogenic mutant while maintaining the pathogenic fraction. The egg, on the other hand, selects reciprocally. In support of this is the fact that results were similar in other experiments, in which the same technique resulted in the attenuation of the virus of Newcastle disease.[2] Following manipulation of the Newcastle disease culture, the apathogenic fraction was obtained in pure form.[1] Whether the mutation was spontaneous or induced by the tissue-culture techniques cannot be answered now.

SUMMARY

A method is reported of propagating the AIS agent in tissue culture composed of Simm-Sanders medium containing fragments of chicken embryo tissue.

Serial passage of the AIS agent in the culture modified the agent between the 10th and 14th passages. The modified culture was apathogenic for chickens, and asymptomatic birds were resistant to challenge with virulent cultures. Subculture of the apathogenic agent through chicken embryos resulted in reversion to the virulent form.

Adding sucrose to a final concentration of 0.4 percent protected the tissue-culture-propagated agent against instability under freezing and storge for at least 66 days.

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STUDIES ON THE FAHEY-CRAWLEY VIRUS

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THE role of agents other than pleuropneumonia-like organisms (PPLO) in the etiology of chronic respiratory disease of chickens and infectious sinusitis of turkeys has not been well established. Fahey and Crawley[3] isolated a viral agent from four out of 52 egg yolk strains believed to be PPLO. In addition, they made five isolations of the same virus from a field outbreak of chronic respiratory disease of chickens and ascribed the etiology of this disease to the virus isolated, on the basis of infectivity studies. Fahey reported the isolation of a similar virus from ducks[4] suffering from chronic respiratory disease and turkeys[5] suffering from infectious sinusitis. He suggested a common etiological factor in the causation of these diseases on the basis of the identical characteristics of the viruses isolated from these birds. Some of the physical properties of the virus have been reported.[15,16]

Johnson and Domermuth[12] studied the infectivity of this agent for chickens and turkeys, when administered alone, along with PPLO, and in conjunction with *Escherichia coli*. They concluded that the Fahey-Crawley virus would initiate more severe symptoms and lesions in birds harboring PPLO, and that in addition, certain strains of *E. coli* or perhaps other organisms may incite a severe disease like the air-sac disease that occurs under field conditions. The role of *E. coli*

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in chronic respiratory disease has been further substantiated by Gross.[7,8]

Grumbles *et al.*,[10] working with a known strain of the Fahey-Crawley virus reported that the virus failed to cause recognizable infection in turkeys inoculated into the sinus with the virus. Also, the virus was not isolated from any group of turkeys with gross lesions of infectious sinusitis.

Although evidence has been presented that the Fahey-Crawley virus may play an etiological role in chronic respiratory disease and infectious sinusitis, it was felt that further work was necessary to establish this fact. With an ultimate view of establishing the role of this agent in these diseases, a preliminary study was undertaken to establish the ability of this virus to incite chronic respiratory disease in chickens of different age groups, and infectious sinusitis in turkeys.

MATERIALS AND METHODS

Day-old New Hampshire chicks were obtained from a U.S. Pullorum-Typhoid Clean hatchery and were housed separately in an isolation unit. The chicks were taken from here for experimental use as day-old, 3-week-old, and 6-week-old birds. Two such lots of day-old chicks furnished the experimental birds for the experiment.

Thirty 6-week-old turkeys, purchased as day-old poults from a U. S. Pullorum-Typhoid Clean hatchery and housed in an isolation unit, were used in the experiments along with the 6-week-old chickens.

Stock virus. The 16th egg passage of the Fahey-Crawley virus was obtained from Dr. Salsbury's Laboratories. It was treated with 500 units of penicillin, and 1 mg of streptomycin per ml and passed once by the chorioallantoic sac (CAS) route in 10-day-old chicken embryos. Chorioallantoic membranes (CAM) and allantoic fluid were harvested from living eggs on the 4th day post-inoculation and a 10 percent suspension made in sterile phosphate buffer solution of pH 7.0 $\pm$ 0.1. The stock virus was centrifuged at 2500 rpm for 10 minutes and the supernatant distributed in 5 ml amounts in sterile vials. The embryo lethal dose$_{50}$ (ELD$_{50}$) was estimated

and found to be 0.1 ml x $10^{-4.5}$. The suspension was treated with the antibiotics the same way and stored at –20 C until used.

Fertile eggs. The re-isolation and propagation of the virus was accomplished in 10-day-old embryonated chicken eggs from a non-immune source.

EXPERIMENTAL PROCEDURE

Experiment 1. Forty day-old chicks were inoculated intratracheally with 0.1 ml of the 10 percent suspension of the virus and put in brooder batteries in a separate unit. On the same day, three uninoculated chicks were killed and their lungs, trachea, liver, spleen and blood were collected in sterile containers in three pools as follows: (1) lungs and trachea, (2) liver and spleen, and (3) blood. During the following 10 days, three chicks of the inocuated group were killed every 24 hours beginning at the time of inoculation of the test group, and their tissues and blood similarly collected. The chicks were examined daily for respiratory symptoms.

A 10 percent suspension of each pool of tissues except blood was made, using sterile mortar and pestle and then centrifuged at 2500 rpm for 10 minutes. The supernatant was poured off in sterile tubes and 500 units of penicillin and 1 mg of streptomycin were added per ml of the suspension and blood. After 1 hour of incubation at room temperature, 0.2 ml from each pool was inoculated by the CAS route into each of five eggs. The eggs were candled every 24 hours. The embryos dying within the first 24 hours were discarded as death due to trauma and those dying thereafter were opened and the embryos and CAM examined for lesions. Allantoic fluid and CAM were harvested from living embryos on the 4th day post-inoculation and passed again into 10-day old chicken embryos after treatment with antibiotics. The isolation of the virus was considered positive under the following circumstances: A) death of the chicken embryos between 3 and 6 days post-inoculation and the mortality in chicken embryos reproduced on further passages, B) presence of characteristic lesions in the embryos of the Fahey-Crawley virus such as pock lesions

on the CAM, hemorrhages and dwarfing of the embryo, and foci of necrosis in the liver, C) development of a significant serum neutralization (SN) titer for the Fahey-Crawley virus in the sera of the inoculated group, and its absence in the control group, D) failure of the sera of the inoculated group to give positive PPLO serum plate test, and absence in their sera of antibodies for Newcastle disease and infectious bronchitis.

The presence of PPLO in the tissues of each group of birds was eliminated by culturing a portion of the lungs and trachea in PPLO broth (Difco). Blood was withdrawn from three inoculated birds and three control birds 40 days after inoculation, and the sera from each group were pooled together for determinations of SN titer to the Fahey-Crawley and infectious bronchitis viruses, hemagglutination-inhibition titer to Newcastle disease, and the serum plate test for PPLO.

Experiment 2. Thirty, 3-week-old chickens were inoculated with 0.2 ml of a 10 percent suspension of the viral agent. Tissues and blood were collected from two birds killed each day from 24 hours to 12 days following the day of inoculation. Two control birds were also killed on the day of inoculation, and on 5th and 12th days and their tissues and blood collected. Blood was withdrawn from three inoculated birds and three control birds, 40 days after inoculation, to be used for estimation of antibody level as in experiment 1.

Experiment 3. Thirty, 6-week-old chickens, and twenty-two, 6-week-old turkeys were inoculated with 0.2 ml of the virus suspension. The numbers of chickens, and the days they were killed, and the collection of sera for estimation of antibody level were the same as in the previous experiment. Two turkeys were killed from 1 to 5 days and then on days 7, 9, and 11 post-inoculation.

Experiment 4. Forty, day-old chicks were inoculated with 0.1 ml of the stock virus. Three chicks were killed each day from one day up to 11 days post-inoculation, and then on 13th and 15th day. Tissues and blood from control birds were also collected on the initial day and on the 5th and 15th days. Blood for estimation of antibody level to the agents listed

TABLE I

The recovery of the Fahey-Crawley virus from different tissues and the serological response induced after intratracheal inoculation of the virus*

Experiment No.	Birds Inoculated	Tissues	Days after inoculation virus re-isolated															Average log neut. index 40 days post-inoculation test	control
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	Day-old Chicken	Lungs and Trachea	—	+	+	+	+	+	+	+	+	+	+	..	..	..	..	..		
		Liver and Spleen	—	+	+	+	+	—	—	—	—	—	—	..	..	..	..	..	2.8	1.0
		Blood	—	+	+	+	+	—	—	—	—	—	—	..	..	..	..	..		
4	Day-old Chicken	Lungs and Trachea	—	+	+	+	+	+	+	+	+	—	—	—	..	—	..	—		
		Liver and Spleen	—	+	+	+	+	—	—	—	—	—	—	—	..	—	..	—	2.5	1.1
		Blood	—	+	+	+	+	—	—	—	—	—	—	—	..	—	..	—		
2	3-week Chicken	Lungs and Trachea	—	+	+	+	—	—	—	—	—	—	—	—	—	..	..	..		
		Liver and Spleen	—	+	+	—	—	—	—	—	—	—	—	—	—	..	..	..	2.3	1.3
		Blood	—	+	+	—	—	—	—	—	—	—	—	—	—	..	..	..		
3a	6-week Chicken	Lungs and Trachea	—	+	+	+	—	—	—	—	—	—	—	—	—	..	..	..		
		Liver and Spleen	—	+	+	—	—	—	—	—	—	—	—	—	—	..	..	..	2.3	1.1
		Blood	—	+	+	—	—	—	—	—	—	—	—	—	—	..	..	..		
3b	3-week Turkey	Lungs and Trachea	—	+	+	+	—	—	..	—	..	—	..	—	..	..	..	..		
		Liver and Spleen	—	+	+	—	—	—	..	—	..	—	..	—	..	..	..	..	2.4	1.2
		Blood	—	+	+	—	—	—	..	—	..	—	..	—	..	..	..	..		

* + = positive re-isolation; — = no re-isolation; .. = no tissue collected

above was collected as in previous experiments.

Microscopic sections of the tissues of chicks collected on 5th, 10th and 14th day after the inoculation of the virus, and also sections of the liver and heart of the chicken embryos used for re-isolation were made and examined.

RESULTS

Table 1 and table 2 summarize the data collected for the series of experiments. It will be seen (table 1) that the virus could be re-isolated from the day-old birds (experiment 1) up to 10 days after inoculation and up to 4 days from the liver, spleen and blood. Birds were not available for re-isolation studies after the 10th day. From the birds inoculated in the 3-week and 6-week-old groups, the virus could not be re-isolated from the lungs and trachea after 3 days, and beyond 2 days post-inoculation, from the liver, spleen, and blood.

Table 2 shows the symptoms observed in the inoculated birds. A slight nasal discharge was observed in the chicks (experiment 1) from the 2nd up to the 10th day when the last of the inoculated birds were killed. The chicks in experiment 4 developed this discharge on the 2nd day and continued to have a discharge up to the 7th day. The nasal discharge was observed only from 3 to 6 days post-inoculation in the 3-week-old chickens and not at all in the 6-week-old chickens. It will be seen that the turkey poults developed this discharge on the 2nd day post-inoculation and continued to have it up to 15 days. It was noticed that the discharge was in moderate amount and tended to be mucoid in nature. Some mucous discharge and slight congestion of the eyes was noticed.

DISCUSSION

The results obtained would indicate that the virus did not have the ability to produce any serious respiratory distress in the chickens except perhaps a mild inflammatory reaction and a slight nasal discharge. A specific type of cellular reaction was evident from the microscopic sections of the lungs of 5 and 10 day-old inoculated chicks (experiment 4) that showed many lymphofollicular areas in contrast to very few

TABLE II

The onset and duration of nasal discharge in different groups after intratracheal inoculation with Fahey-Crawley virus

Experiment number	Birds inoculated	Days after inoculation when nasal discharge noticed																	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Day old Chicken	−	−	+ −	+	+	+	+	+	+	+ −	+ −	−	−	−	−	−	−	−	−
4	Day old Chicken	−	−	+ −	+	+	+	+ −	+ −	−	−	−	−	−	−	−	−	−	−	−
2	3-week Chicken	−	−	−	+	+	+ −	−	−	−	−	−	−	−	−	−	−	−	−	−
3a	6-week Chicken	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3b	3-week Turkey	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−

+ = Nasal discharge
± = Suspicious nasal discharge
− = No nasal discharge

such areas in the lungs on un inoculated chicks of the same ages. Further, the length of time the virus could be re-isolated from the respiratory tissues of the chicks would seem to suggest multiplication of the virus rather than passive recovery of the agent. The re-isolation of the virus from the blood, liver and spleen, up to 4 days after inoculation, and from the lungs and trachea up to 7 to 10 days, suggested that there was a viremia and that the specific site of multiplication of the virus was in the lungs.

The isolation of the virus up to 3 days from the lungs and trachea of the 3-week-old and 6-week-old birds and the failure to isolate the agent beyond this period from any tissues of the birds of this age group may mean that the birds of these age groups were not as susceptible as young chicks. The inability of the virus to multiply was not due to the presence of circulating antibodies in the blood because of the insignificant SN titers to this agent in the sera of these birds before the inoculation of the agent.

A further evidence of mild infection with this virus was provided by an SN index of over a 100 neutralizing ELD_{50} obtained with the sera of the inoculated groups as against SN index of below 32 observed in sera from the control group.

The continued nasal discharge observed in the turkey poults seemed to be suggestive of infection with the virus, considering the fact that their sera were negative to the PPLO serum plate test before the inoculation of the virus and PPLO could not be cultured from their tissues at any time. However, the sera of these birds became positive to the PPLO serum plate test, when tested after 40 days. Since the inability to isolate PPLO from birds suffering from infectious sinusitis does not necessarily eliminate an infection by this agent, it was assumed that the prolonged nasal discharge and congestion and watering of the eyes was due to infection with PPLO rather than the Fahey-Crawley virus, in view of the inability to isolate the virus from the tissues of these birds, and the development of positive serum plate test to PPLO. Johnson and Domermuth[12] reported a similar observation in turkeys negative to PPLO freeze-agglutination test (F-A) which be-

came positive to this test after administration of the Fahey-Crawley virus. They suggested that it was possible that the birds were harboring PPLO in a latent form and not reacting to the F-A test at the onset, and that the virus activated the PPLO.

Chicken embryos infected with the Fahey-Crawley virus frequently showed macroscopic areas of necrosis of the liver that were more prominent near the edges of the lobes. Microscopic sections revealed a focal type of necrosis. Oftentimes malformations of the liver was noticed with an entire side of the liver reduced to a tiny mass.

There seems no question about the ability of the virus to induce specific lesions and death of the chicken embryo, but whether or not it has the ability to produce clinical signs of disease in the adult birds remains to be fully established. The fact that the virus could be re-isolated for a longer period of time from young chicks than older birds, and also the appearance of a nasal discharge in them, indicates that the young chicks are more susceptible to the virus than the older birds. The ability of the virus to cause lesions and death of chicken embryo indicates that the virus does have pathogenic properties.

The role the Fahey-Crawley virus plays in the etiology and pathogenesis of the chronic respiratory disease of chickens and infectious sinusitis of turkeys needs to be investigated further. Available evidence indicates that the virus is capable of producing a mild, inapparent, respiratory infection, and that it may activate a latent PPLO infection. Although evidence that the virus may activate a latent PPLO infection has been obtained in these experiments, and also reported by Johnson and Domermuth,[12] further research into this problem needs to be done before accurately establishing the role of the virus in the chronic respiratory disease of chickens and infectious sinusitis of turkeys. It has been shown that other agents such as *E. coli*, the ornithosis virus, and the infectious bronchitis virus may also play a role in the activation of these diseases.

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SUMMARY

1. Day-old, 3-week-old and 6 week old chickens and 3-week-old turkeys were infected with Fahey-Crawley virus. The symptoms, length of time the virus could be re-isolated, and the serological response induced were studied.

2. Only a mild nasal dischage was observed in the inoculated birds of the day-old and 3-week-old groups. Six-week-old birds did not show any nasal discharge or other symptoms.

3. The virus could be re-isolated from the day-old chicks up to 10 days after inoculation of the virus. The virus could not be isolated from the birds of the 3-week-old and 6-week-old groups later than 3 days after inoculation of the virus.

4. The serological titers determined at 40 days after inoculation of the virus were over a 100 neutralizing ELD_{50} doses whereas those of the control were below 32.

5. The respiratory symptoms observed in the turkey poults seemed to be due to infection with pleuropneumonia-like organisms and it seemed likely that the Fahey-Crawley virus activated a latent PPLO infection.

6. Specific liver necrosis was observed frequently in chicken embryos infected with the Fahey-Crawley virus. Microscopic sections revealed a focal type of necrosis.

7. The ability of the virus to induce an infection by itself and its role in the chronic respiratory disease of chickens and infectious sinusitis of turkeys are briefly discussed.

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TREATMENT OF EXPERIMENTAL INFECTIOUS SINUSITIS OF TURKEYS WITH ERYTHROMYCIN

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PLEUROPNEUMONIA-like organisms (PPLO) have been implicated as a cause of infectious sinusitis (IS) of turkeys. The literature on this subject has been reviewed by other authors.[2,5,6] Reports on treatment of IS with erythromycin have been made by Hamdy, *et al.*[2,3] and Holper, *et al.*[5] This paper represents the results of erythromycin treatment of infectious sinusitis induced in young turkeys by the intrasinus injection of avian PPLO.

MATERIALS AND METHODS

Strains. Strains of PPLO used in these experiments were No. 295 (S-6, Adler, California); No. 455 (678, Hofstad, Iowa); No. 540 (PM-18, Osborn, Minnesota); No. 1990 (C-2, Grumbles, Texas); No. 1991 (T-2, Grumbles, Texas); and No. 2453 (734, Hofstad, Iowa).

Infection of turkeys. Turkeys were obtained from a commercial source at one day of age and raised in isolation in brooder houses at our station. At 6 to 8 weeks of age, both sinuses of each turkey were injected with one ml of PPLO culture prepared in embryonating chicken eggs as described in a previous publication.[9] The culture was usually found to contain approximately 10^5 PPLO per ml as measured by phenol red broth and agar plate dilution methods. The percentage of birds which developed sinus swelling varied among groups

of turkeys with the same or different strains of PPLO. Overall, from 55 to 96 percent of the turkeys showed swelling at the time of treatment, which was usually 2 weeks after inoculation.

Treatment and evaluation. Both sinuses of each turkey were treated once by hypodermic puncture through the skin. Three forms of erythromycin were used: erythromycin base, erythromycin thiocyanate, and erythromycin glucoheptonate. The amount of sinus swelling of each turkey was determined individually at weekly intervals for 4 weeks and less often thereafter. In early experiments, observations were continued for as long as 14 weeks to allow ample time for possible recurrence of sinus swelling. Birds were scored negative when no swelling was visible. Those turkeys with slight to marked swellings were considered positive. In order to compare results obtained when erythromycin was administered at different times post infection, the infected control group was treated with erythromycin base 4 weeks later than the other treatment groups in experiments 1 and 2.

Culturing sinus exudates for PPLO. Attempts were made to isolate PPLO by culture from sinus exudates as well as from swab specimens from normal appearing sinus cavities. All specimens were placed in chicken infusion broth[4] which was modified by the addition of 0.5 percent dextrose, 0.018 percent phenol red and replacement of 20 percent chicken serum by 1.5 percent PPLO serum fraction. Thallium acetate, 0.5 gm per liter, and penicillin, 1000 units per ml., were added as bacterial inhibitors. Cultures were incubated at 37 C and transferred every 4 days. They were carried through five passages before being declared negative for PPLO growth. A culture was judged positive when typical PPLO colonies were observed on the surface of agar (PPLO agar, Difco., plus 1.5% PPLO serum fraction, Difco.) after at least 4 days incubation.

Serology. Blood serum samples were collected from all turkeys at the time of PPLO inoculation. At the time of treatment and during the experiment, samples were taken from a

representative number of each treatment. They were tested by the PPLO hemagglutination inhibition test (1), with PPLO antigen obtained from Connaught Research Laboratories. To avoid false negatives due to the pro-zone phenomenon, serums were diluted to at least 1-40. A specimen was considered positive when complete inhibition occurred at a dilution of 1-10 or more.

Antibiotic sensitivity. Sensitivity of the strains of PPLO to antibiotics was determined in one test *in vitro* by serial two-fold dilutions of the antibiotic in Grumbles' phenol red broth[7] followed by inoculation with the PPLO to be tested. After 24 hours incubation at 37 C, 0.25 ml of the culture was transferred to a new tube of the same medium without antibiotic and incubated at 37 C for 7 days to detect growth of the organisms. When possible, the sensitivity of the original strain and of one or more strains reisolated from turkeys, which had been inoculated with that strain and treated, was determined.

RESULTS

Experiment 1. Strain 540. Serums from all turkeys at the time of PPLO infection were negative for PPLO HI antibodies. Attempts to culture PPLO from tracheal swabs and sinus wash specimens collected from 10 percent of the birds were negative. At treatment, nine of nine sinus exudate specimens cultured were positive for PPLO.

Data on the reduction in swelling produced by the various treatments may be seen in table 1. Examination of the sinuses immediately following treatment showed all three forms of erythromycin to be irritating to some extent, as judged by swelling and redness. Erythromycin glucoheptonate appeared to be the most irritating and erythromycin thiocyanate the least irritating. In all cases inflammation had disappeared by 48 hours post treatment. Some sinus swelling recurred in the treated groups during the period of 4 to 14 weeks post treatment. It was most prominent in the groups treated with erythromycin thiocyanate and erythromycin glucoheptonate. Of two swab specimens collected from each group, both were positive for PPLO from the erythromycin glucoheptonate

group and one was positive from each of the control and erythromycin thiocyanate groups; no isolations were made from the erythromycin base treated groups.

Due to technical difficulties, the results of the PPLO HI tests on serums collected at the termination of the experiment were not accurate and are not included in this paper.

TABLE I

Treatment of experimental PPLO sinusitis induced with strain 540 in young turkeys with three forms of erythromycin. (Experiment 1)

Treatment[1]	Number of turkeys	Sinus Swelling		
		Weeks after treatment	Percent affected	Percent change
Control	40	0	84	
		2	90	
		4	79	
Erythromycin base[2]		9	33	
50 mg / sinus		14	35	-58
Erythromycin	38	0	97	
thiocyanate		2	36	
38 mg / sinus		4	34	
		14	41	-58
Erythromycin	41	0	86	
glucopheptonate		2	33	
50 mg / sinus		4	12	
		14	20	-76
Erythromycin base	41	0	97	
47 mg / sinus		2	4	
		4	2	
		14	5	-94

[1] Turkeys in each group were infected at 8 weeks of age and treated at 10 weeks of age.
[2] Infected control group which was treated 4 weeks after initiation of the experiment, or at 14 weeks of age.

Experiment 2. Strain 295. At the time of PPLO infection, all turkeys were negative to the PPLO HI test, and 23 tracheal swabs were negative of 25 cultured for PPLO. Four saline washes from normal appearing sinuses were all negative for PPLO. At treatment, 24 of 25 samples of sinus exudate yielded PPLO on culture. Of 25 serum specimens examined by the PPLO HI test, 24 were positive.

Data on the change in the percent swelling produced by various treatments are shown in table 2. Erythromycin thiocyanate caused moderate local swelling and redness for the first 24 hours following treatment. Erythromycin base and erythromycin glucoheptonate both caused an inflammatory reaction which mostly subsided within 48 hours.

TABLE II

Treatment of experimental sinusitis induced with strain 295 in young turkeys with three forms of erythromycin. (Experiment 2)

Treatment[1]	Number of turkeys	Sinus Swelling		
		Weeks after treatment	Percent affected	Percent change
Control	48	0	98	
		2	94	
		4	92	
Erythromycin base[2] 50 mg / sinus		12	30	-69
Erythromycin thiocyanate 50 mg / sinus	48	0	98	
		2	33	
		4	31	
		12	33	-66
Erythromycin glucoheptonate 50 mg / sinus	45	0	96	
		2	29	
		4	16	
		12	30	-69
Erythromycin base 50 mg / sinus	44	0	100	
		2	20	
		4	26	
		12	16	-84

[1] Turkeys in each group were infected at 6 weeks of age and treated at 10 weeks of age.
[2] Infected control group which was treated 4 weeks after initiation of the experiment, or at 14 weeks of age.

Results of the PPLO HI test conducted four weeks after infection showed 100 percent reactors, and a similar percentage of reaction in all treatment groups at the termination of the experiment. PPLO were isolated from most exudates from swollen sinuses in the various groups 4 and 9 weeks post treatment. No later isolations were attempted. Of specimens from non-swollen sinuses, one of four from each of the erythromycin thiocyanate and erythromycin glucoheptonate treated

groups, none of four from the erythromycin base group, and two of four from the control group were positive.

Experiment 3. Strain 540. Immediately prior to PPLO infection, all turkeys were negative to the PPLO HI test, and PPLO were isolated from six of the thirty tracheal swabs cultured.

PPLO strain 540 was apparently very pathogenic, since about one-third of the turkeys died within 3 weeks post inoculation. Respiratory and central nervous system signs, together with air sac lesions were noted. No pathogenic agent other than PPLO was isolated, nor were any other indications of disease observed, so it was presumed that the PPLO were the cause of death. Of 20 blood samples collected at the time of treatment, 17 were positive to the PPLO HI test. PPLO were isolated from all sinus exudates.

Data on the reduction of sinus swelling following erythromycin treatment are shown in table 3. Four weeks post treatment, only one of 189 turkeys tested was negative to the PPLO

TABLE III

Treatment of experimental sinusitis induced with strain 540 in young turkeys with two forms of erythromycin. (Experiment 3)

		Sinus Swelling		
Treatment[1]	Number of turkeys	Weeks after treatment	Percent affected	Percent change
Control	43	0	77	
		2	88	
		4	91	+18
Erythromycin glucoheptonate 50 mg / sinus	36	0	72	
		2	22	
		4	39	—46
Erythromycin glucoheptonate 10 mg / sinus	35	0	94	
		2	46	
		4	60	—36
Erythromycin base 50 mg / sinus	36	0	81	
		2	3	
		4	3	—97
Erythromycin base 10 mg / sinus	43	0	67	
		2	35	
		4	47	—31

[1] Turkeys in each group were infected at 7 weeks of age and treated at 10 weeks of age.

HI test, and there was a recurrence of sinus swelling. In each of the two groups treated with 10 mg per sinus, three of six swabs from non-swollen sinuses yielded PPLO on culture. Of the two groups treated with 50 mg per sinus, one of six non-swollen sinuses was positive from the erythromycin base treated group, and none were positive from the erythromycin glucoheptonate group. Four of four control group specimens were positive for PPLO.

Experiment 4. Strain 455. At the time of PPLO infection, blood serums from ten percent of the turkeys were negative for the PPLO HI test and tracheal swabs from these same turkeys were negative for PPLO. At treatment, PPLO HI tests and attempts to isolate PPLO from sinus exudate were conducted on specimens from 10 percent of the turkeys. All were positive.

Data on the reduction of sinus swelling following treatment are shown in table 4. Some irritation was produced by both polyethylene glycol (PEG) and the PEG containing erythromycin base, but in all instances it was transitory and all evidence of swelling was gone in 24 hours. PPLO were cul-

TABLE IV

Treatment of experimental sinusitis induced with strain 455 in young turkeys using polyethylene glycol and erythromycin base in polyethylene glycol. (Experiment 4)

		Sinus Swelling		
Treatment[1]	Number of[2] turkeys	Weeks after treatment	Percent affected	Percent change
Control	10	0	70	
	8	2	75	
	8	4	75	+ 7
PEG[3] 200	20	0	70	
1 ml / sinus	18	2	89	
	18	4	50	—29
Erythromycin	20	0	70	
base in PEG	18	2	22	
50 mg / sinus	17	4	0	—100

[1] Turkeys were infected at 7 weeks of age and treated at 9 weeks of age.
[2] One turkey sacrificed from control group and two from each treated group for assay purposes. One turkey died in control group and one in group treated with erythromycin in PEG.
[3] PEG = polyethylene glycol.

tured from sinus exudate at 4 weeks post treatment from the untreated control and the PEG treated groups. No specimens were taken from non-swollen sinuses.

Experiment 5. Strains 1990, 1991, and 2453. Immediately prior to PPLO infection, all of 15 turkeys tested were negative to the PPLO HI test. One of 15 tracheal swabs yielded PPLO on culture. The infection caused by strain No. 2453 appeared to be more severe than that by strains 1990 or 1991, since sinus swelling appeared most marked in turkeys inoculated with this strain. At treatment time blood samples and sinus exudate specimens were collected from four turkeys of each group. All but one of the turkeys reacted to the PPLO HI test. Five of eight cultures from the various groups yielded PPLO.

Information on the reduction of sinus swelling following treatment is shown in table 5. Irritation produced by the treatment was mild and all swelling was gone within 24 hours.

TABLE V

Treatment of experimental sinusitis induced by strains 1990, 1991, and 2453 in young turkeys using erythromycin base in PEG. (Experiment 5)

			Sinus Swelling		
Strain	Treatment[1]	Number of turkeys	Weeks after treatment	Percent affected	Percent change
1990	Control	17	0	59	
			2	77	
			4	77	+24
1990	Erythromycin base 50 mg / sinus	18	0	56	
			2	21	
			4	7	—87
1991	Control	18	0	50	
			2	64	
			4	100	+50
1991	Erythromycin base 50 mg / sinus	18	0	44	
			2	43	
			4	57	+22
2453	Control	18	0	94	
			2	94	
			4	90	— 4
2453	Erythromycin base 50 mg / sinus	18	0	100	
			2	79	
			4	85	—14

[1] Turkeys were infected at 6 weeks of age and treated at 8 weeks of age.

The results of culturing sinus exudate at the end of the experiment were not satisfactory due to heavy bacterial contamination.

Erythromycin sensitivity tests. Measurements of the *in-vitro* sensitivity of the various cultures of PPLO, before and after contact with erythromycin in the infected turkey sinus, are shown in table 6. It was necessary to presume that the re-isolated strains were the same as the inoculated strain since methods of differentiation were not available at the time of these experiments. There did not appear to be any marked tendency for development of resistance to erythromycin, which was consistent with the findings of Osborn, *et al.*[8]

DISCUSSION

These experiments confirm the findings of other workers[2,3,5] that erythromycin is effective in the treatment of IS. The results in field cases will probably depend upon the sensitivity of the strain of the PPLO and the time which elapses between the occurrence of swelling and treatment. Both the *in vivo* and the *in vitro* data indicate that strain variation occurs. Treatment of the infected control turkeys, 4 weeks later than the other groups in experiments 1 and 2, demonstrated reduced efficacy of erythromycin in the more chronic condition, and indicated the need for repeated treatment in chronic cases. While time did not allow for a large number of attempts to culture PPLO from sinuses following treatment, the persistence or recurrence of swelling did seem to accompany the presence of PPLO. Thus, it appeared that elimination of the PPLO from the sinuses was necessary to prevent recurrence of the sinus swelling.

There is obviously a problem in differentiation of recurrence of swelling of a sinus from which the PPLO have not been eliminated and that due to a new infection. In this study, it was presumed that obvious swelling within 2 weeks post treatment was due to failure to eliminate the infection. This was in part substantiated by more PPLO isolations, at 4 weeks post treatment, from non-swollen sinuses in groups where greater recurrence of sinus swelling was seen.

TABLE VI

Sensitivity of PPLO strains to erythromycin *in vitro* before and after *in vivo* erythromycin treatment

Experiment Number	Strain Number	Minimum concentration, mcg./ml.		
		Erythromycin thiocyanate	Erythromycin glucoheptonate	Erythromycin base
1	540	0.312/——[1]	0.125/——[1]	0.078/——[1]
	685[2]	0.156/——	0.625/——	0.078/——
	689[2]	0.156/——	0.625/——	0.156/——
2	295	0.245/0.245	0.245/0.245	0.245/0.245
	915[3]	0.49 /3.91	0.49/ 7.82	0.49/ 7.82
	918[3]	0.49 /0.49	0.29 /1.96	0.49 /0.98
3	540	15.62/15.62	1.96 /15.62	3.91 /15.62
	1017[4]	1.96/15.62	15.62/15.62	indefinite/ 3.91
	1014[4]	0.49/ 3.91	0.98 / 7.82	0.49 / 7.82

[1] Inhibiting concentration/lethal concentration.
[2] PPLO strain reisolated from sinus inoculated with strain 540.
[3] PPLO strain reisolated from sinus inoculated with strain 295.
[4] PPLO strain reisolated from sinus inoculated with strain 540.

Studies on serum samples from infected turkeys throughout the experiments with the PPLO HI test showed that intrasinus treatment had not prevented antibody production. All turkeys in all groups were found to have a significant titer within 4 to 6 weeks after infection.

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CONCLUSIONS

Sinusitis was induced in young turkeys by the intrasinus injection of strains of avian pleuropneumonia-like organisms (PPLO) and subsequently treated with various dosages of three forms of erythromycin. Turkeys infected with four strains of PPLO responded to erythromycin therapy of 50 mg per sinus, while those infected with two other strains did not, as judged by sinus swelling and reisolation of PPLO. Erythromycin base was more effective than either erythromycin thiocyanate or erythromycin glucoheptonate. Treatment at 2 weeks after infection was more effective than that at six weeks.

The recurrence of sinus swelling in some erythromycin treated groups seemed to correlate with isolation of PPLO from non-swollen sinuses of that group. There were no indications of development of resistance by the PPLO to erythromycin as a result of contact of the organism to a single treatment of the infected birds with the drugs.

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RESPONSE OF BIRDS TO ONE INTRANASAL VACCINATION WITH B-1 STRAIN OF NEWCASTLE DISEASE VIRUS

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SINCE the original description and application of the B-1 strain of Newcastle disease (ND) virus,[9,10,11] considerable experimental data have been gathered on the serological and immune responses of birds vaccinated with this strain.[6,12,13,15,19,20] Most of the authors concerned themselves with the response of chicks vaccinated at an early age.[4,5,7,13,14] Bankowski *et al.*[2] studied the response of 2-month-old susceptible chickens to ND B-1 strain vaccine administered in

the drinking water. Doll *et al.*[7] demonstrated a better immune response in birds vaccinated at 5 weeks of age than in those vaccinated at 5 days of age.

This paper reports on the duration of immunity in birds vaccinated once at 2 months of age with a commercially available ND vaccine, B-1 strain. At this age congenital passive immunity against ND has disappeared and the birds are sufficiently developed to respond satisfactorily to the vaccine. The time of appearance and decline of the hemagglutination inhibiting (HI) antibodies, determined on an individual basis, and the relationship between the serum-neutralization (SN) titer and behavior of the birds to an ND intramuscular challenge were further objectives of this study.

MATERIALS AND METHODS

Source of the experimental birds. Fifty-two White Leghorn male chickens were used in this experiment. They were obtained as day-old chicks from a commercial hatchery, situated in an area where the poultry population is heavy and ND vaccination is extensively practiced. The dams were vaccinated against ND by the stick method at 6 weeks of age and revaccinated intramuscularly at 14 weeks of age. The day-old chicks were raised under isolation until the experiment began. Challenge tests of randomly selected birds from this group showed them to be susceptible to ND and infectious bronchitis.

The vaccine strain. The vaccine was obtained directly from the manufacturer and maintained at 2 C after arrival. It was reconstituted according to the instructions of the manufacturer, and its examination yielded no bacterial or viral contaminants. The vaccine, titrated in five embryonating chicken eggs per dilution, had an ELD_{50} of 10^{-8} per ml.[18]

Isolation facilities. At 2 months of age, the experimental birds were banded and placed in a movable metal cage on a field approximately a mile from any known poultry premises. During their contacts with the birds, the experimenters wore sterilized garments and avoided any previous contact with infected material. A separate caretaker was

assigned to this group of birds.

Climatic conditions during winter. The experiment was initiated in mid-December. The average maximum temperature for December was 52.2 F and the average minimum temperature for the same month was 38.5 F. The averages of the maximum and minimum temperatures for January were 52.6 F and 38.2 F, respectively. The rainfall for December was 2.97 inches and that for January was 4.91 inches.

Experimental procedures. Two days before vaccination all experimental birds were bled by heart puncture for HI[3] and PPLO-agglutination tests.[1] The vaccinated birds were bled on the 4th, 7th, 10th, 17th, 26th, 41st, 57th, and 79th day after vaccination.

One drop of the reconstituted vaccine was administered intranasally to each bird. One month after vaccination, 10 additional White Leghorns, of comparable age and susceptible to ND were bled for an HI test and placed in the same cage. These birds served as controls for any adventitious infection from the field.

At the end of the 4th, 5th, and 6th month after vaccination, 10 randomly selected principals along with two field controls were removed from the premises, placed in an isolation unit, bled for serological studies, and challenged intramuscularly with 200,000 ELD_{50} of GB (Texas) strain ND virus. At 7 and 8 months after vaccination, only five principals were available for each challenge test. Two field controls were also challenged each time. The inocuated birds were observed for 12 days. If the principals remained alive without showing paralysis, they were considered immune to ND. If the field controls succumbed to challenge, their susceptibility indicated absence of an adventitious ND infection.

Serological procedures. The HI procedure used was similar to that described by Beach.[3] The procedure followed in the SN test was similar to that used for ND[17] and infectious bronchitis.[8] The Montana strain of ND virus was used for the SN test. Its ELD_{50} was in the range 10^{-8} to 10^{-9}. The embryonating chicken eggs used in the SN test originated from dams exposed to ND virus.

TABLE I

Individual responses of 2-month-old birds to a Newcastle disease vaccine (B-1 strain)

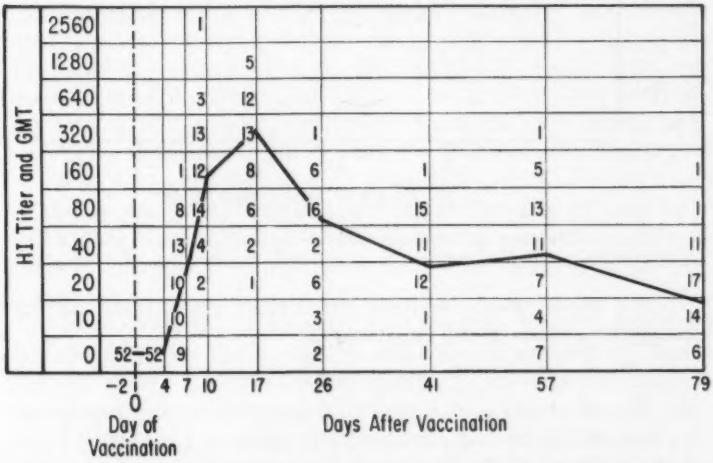
BIRD NO.	79 DAYS AFTER VACC.		BEFORE CHALLENGE		CHALLENGE RESULTS	CHALLENGE RESULTS OF
	HI TITER	SN TITER	HI TITER	SN TITER	OF PRINCIPALS	FIELD EXPOSURE CONTROLS
			CHALLENGE, 16 WEEKS AFTER VACCINATION			
20474	40	10^5	40	10^4	Resistant*	
20475	20	10^5	40	10^4	"	
3638	10	10^3	10	—	"	
3642	20	10^4	40	10^4	"	Susceptible
3649	40	10^5	40	10^5	"	
3665	40	10^5	40	10^5	"	
3682	10	10^3	10	10^2	"	
3688	10	10^4	10	10^3	"	
3691	20	10^4	20	10^3	"	
			CHALLENGE, 21 WEEKS AFTER VACCINATION			
20468	10	10^5	10	10^1	Resistant	
20471	10	10^4	10	10^3	"	
3623	20	10^4	10	10^1	"	
3625	40	10^5	10	10^2	"	
3637	20	10^4	20	10^1	"	Susceptible
3651	10	10^3	—	10^1	"	
3653	10	10^3	10	10^4	"	
3662	0	10^1	0	—	"	
3687	—	—	10	10^1	"	
3689	40	10^4	10	10^4	"	
3696	40	10^4	10	10^3	"	
			CHALLENGE, 25 WEEKS AFTER VACCINATION			
20463	10	10^4	0	10^1	Resistant	
3635	40	10^5	40	10^1	"	
3636	10	10^4	10	10^1	"	
3645	20	10^4	20	10^1	"	Susceptible
3660	10	10^3	20	10^2	"	
3661	20	10^4	20	10^1	"	
3669	10	—	—	10^0	"	
3673	40	10^6	20	10^2	"	
3677	20	10^3	0	10^1	"	
			CHALLENGE, 29 WEEKS AFTER VACCINATION			
20472	40	10^6	0	10^4	Resistant	
20473	20	10^5	—	10^4	"	Susceptible
3674	0	10^1	0	10^1	"	
3684	20	10^4	0	10^3	"	
3697	40	10^4	10	10^4	"	
			CHALLENGE 33 WEEKS AFTER VACCINATION			
20465	20	10^4	20	10^2	Resistant	Susceptible
3639	0	—	0	10^0	"	
3643	20	10^4	0	10^1	"	
3659	40	10^4	10	10^3	"	

*To an intramuscular challenge of 200,000 ELD_{50} of the GB (Texas) strain of Newcastle disease virus.

RESULTS

Table 1 shows that all vaccinated birds were immune to challenge for at least 8 months, the longest period tested. The field controls were consistently susceptible to challenge.

Figure 1 shows an early rise in the HI antibodies. On the 7th day after vaccination, 22 birds had titers varying between 1:40 and 1:80. The HI antibodies reached their peak on the 17th day after vaccination, with a geometric mean



Note: The numbers scattered througout the table indicate the number of birds showing respective HI titers. The curve represents the GMT.

Fig. 1. Response of birds to a Newcastle disease vaccine. Distribution of the hemagglutination inhibition (HI) and geometric mean titers (GMT). The numbers scattered throughout the figure indicate the number of birds showing HI titers. The curve represents the GMT.

titer (GMT)[16] of 320. Nine days later, the GMT fell to 61. From the 26th day until the 79th day, the HI antibody level slowly declined to a GMT of 19.

Figure 2 indicates significant SN titers[21] in two birds as early as the 7th day after vaccination. By the 10th day all seven sera tested had significant levels of SN antibodies, which persisted in all birds until the 4th month. Considerable variations among individuals were observed in the following months (table 1).

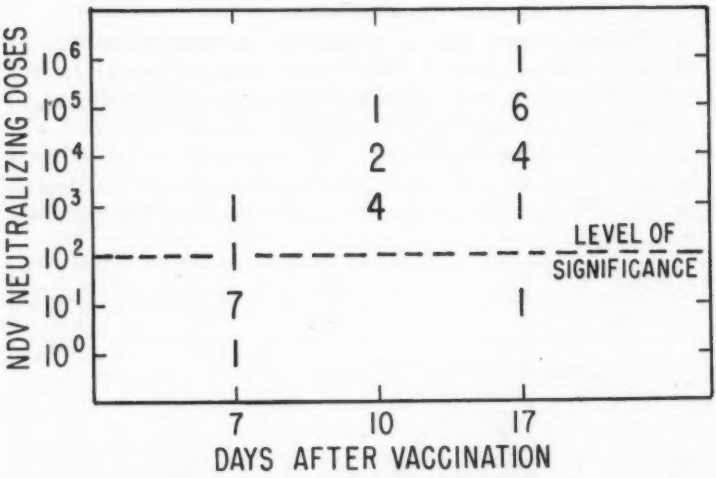


Fig. 2. Number of birds showing Newcastle disease virus (NDV) neutralizing antibodies after one intranasal vaccination (B-1 strain).

All birds were negative for PPLO agglutinins during the entire course of the experiment.

DISCUSSION

In the absence of adventitious exposure, one intranasal application of the B-1 strain of ND virus to 2-month-old susceptible birds resulted in immunity to an intramuscular challenge with a virulent strain of ND virus for at least 8 months following vaccination.

Since immunity is judged by the response of the host to an antigen, it is sometimes dangerous to draw conclusions on the characteristics of the antigen; in reality we observe only the reactions of the host. If the full potential of response of chickens to the B-1 strain of ND virus is hampered either by the immaturity of the host or by a significant level of congenital passive immunity, the immune response of the vaccinated chicks cannot be maximal. The long duration of resistance to challenge observed in this experiment is probably due to the capacity of the host to react satisfactorily to the vaccine.

Figure 1 shows that a rapid and significant rise in HI titer followed vaccination. The HI titers reached their peak approximately 17 days after vaccination and rapidly declined to a GMT varying between 61 and 19. It was interesting to observe such a high (GMT of 320) HI response in birds vaccinated intranasally with the B-1 strain of ND virus. The absence of congenital passive immunity at 2 months of age and the capacity of the host to react to a high titer vaccine may account for this unusual response.

An examination of figure 2 reveals an early appearance of a significant level of ND virus neutralizing antibodies. The time of appearance of the SN antibodies closely approximates that of the HI antibodies. The main difference between the two is that the SN antibodies persisted at a significant level for a much longer period than the HI antibodies (table 1).

Table 1 shows that 79 days after vaccination, the SN titer of the majority of the principals varied between 10^4 and 10^6. Individual variations in the serological response are expected; therefore, an SN titer of 10^1 in two birds is not surprising.

It was interesting to observe at the 5th, 6th, 7th, and 8th month after vaccination that a considerable number of birds did not show significant SN titers but were still immune to an intramuscular challenge. Individual variations in the SN titers at this time after vaccination were expected and actually observed. It is known that birds with significant SN and HI titers are immune to ND. It is also known that birds with insignificant HI titers may be immune to an ND challenge.[12] The results of the experiment confirmed the above information. Furthermore, a comparison between the SN titers before challenge and the challenge results indicated that vaccinated birds may be immune in spite of insignificant SN titers.

Frequently the SN test is used as a criterion to judge immunity to ND. The data reported herein show that the presence of significant SN titers correspond with resistance to an intramuscular challenge with ND virus, but insignificant SN titers did not necessarily represent expected suscepti-

bility to challenge. Current experiments generally confirm the above findings.

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SUMMARY

The response of 52 cockerels to a single intranasal vaccination of the B-1 strain of Newcastle disease virus (NDV) consisted of the following:

1. Resistance to an intramusuclar challenge with the GB strain of NDV for 8 months, the longest period tested.

2. Early rise and rapid decline of the hemagglutination inhibiting (HI) antibodies.

3. Early rise of NDV neutralizing antibodies approximating the time of appearance of the HI antibodies but persisting for a much longer period of time.

A comparison of the serum neutralization (SN) and HI titers before challenge and the response of the birds to challenge clearly indicated immunity to challenge in spite of insignificant SN and HI titers.

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CASE REPORT—AVIAN INFECTIOUS BRONCHITIS IN HAWAII

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INFECTIOUS bronchitis (IB), one of the most important respiratory diseases of poultry, is very common throughout the United States of America. Because of its deleterious effects on egg production and quality, this disease is a serious threat to the poultry industry. Schalk and Hawn[11] first reported the existence of IB in 1931, in North Dakota. Subsequently the disease was reported in other states.[2,3,5] In 1941 Asplin[1] reported its presence in England, Sato *et al.*[10] in Japan in 1955, Bijlenga[4] in the Netherlands in 1956, and in 1956 Petek[8] isolated the virus of IB in Italy. It is the opinion of the writers that IB is much more widely spread throughout the world than is generally recognized. Mildness of the respiratory symptoms and short duration of the disease may account for its lack of recognition and possibly misdiagnosis in many countries. This note reports on the presence of IB in the Hawaiian Islands.

In the fall of 1958, the flocks on two adjoining farms near Hilo, in the island of Hawaii, experienced a respiratory disease that rapidly subsided after treatment with sulfathiazole. A third farm on the same island experienced a similar condition. In the early part of 1959 a poultry flock on the island of Oahu was affected by a respiratory condition. Serum

samples from the 14-week-old birds were negative for hemagglutination-inhibiting antibodies. The owner reported that these birds had been vaccinated against Newcastle disease with the B 1 strain at 10 days of age by the intranasal route.

On March 20, 1959, 270 serum samples from the three farms near Hilo and 15 sera from the flock on Oahu were submitted to this laboratory for serological studies. Only 79 of the Hilo samples were tested. The serum-neutralization (SN) test for IB virus was performed according to the method described by Fabricant[6] and Markham.[7]

The results are tabulated below:

TABLE I

Infectious bronchitis virus-neutralizing antibodies in Hawaii.

Island	No. of sera	IB virus-neutralizing doses
Oahu	11	10^7
	1	10^6
	1	10^5
	2	10^4
Hilo	15	10^7
	14	10^6
	49	10^5 or greater
	1	10^4

It is generally agreed that 100 or more doses of IB virus-neutralizing antibodies per ml of serum represent evidence of previous exposure to IB. The high level of IB virus-neutralizing antibodies in all the sera from the Hawaiian Islands definitely indicates that the examined flocks had been exposed to this disease.

The time at which these flocks were first infected with IB cannot be established with certainty. The high level of antibodies indicates that the Hawaiian strain of IB virus had a good antigenic potential. It was capable of inducing the production of a high level of antibodies neutralizing the egg adapted strain at a titer similar to that observed in birds recovered from a Massachusetts L43 infection. Experimental data[9] on the development of SN titers in birds vaccinated against infectious bronchitis suggest that titers of 10^5 or greater, as observed in the Hawaiian sera, may be expected

five to six weeks after infection and for several months thereafter.

The presence of IB in Hawaii is recognized, and this report adds to the understanding of the geographical distribution of this disease.

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CASE REPORT—POX IN THE MOURNING DOVE IN MARYLAND

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ALTHOUGH trichomoniasis has received attention as a cause of death among mourning doves (*Zenaidura macroura*),

little work has been done on other diseases of this species. Kossack and Hanson[2] reported the occurrence of pox lesions in mourning doves in Illinois. Rosen[3] reported that "pigeon pox" had caused a severe mortality in a mourning dove flock near Yreka, Siskiyou County, California.

A severe outbreak of pox that occurred in a captive flock of mourning doves at the Patuxent Research Refuge further emphasizes the need for more study of this disease as a cause of dove mortality. Twenty-two mourning doves were live-trapped on the Agricultural Research Center, Beltsville, Maryland, in September, 1958. The doves were examined for presence of *Trichomonas gallinae* and, when found to be free of trichomonads, were made the nucleus of a captive dove colony.

A mourning dove that had a small, pink nodule on the left eyelid was captured on the Agricultural Research Center on September 17, 1958. The nodule was excised and the cut surface was treated with tincture of merthiolate. The dove then was added to the dove colony.

A dead mourning dove was found in the dove colony on October 5th. This bird was refrigerated immediately and was examined the following day for evidence of the cause of death. Two small yellowish lesions were found in the back of the oral cavity. As the lesions resembled those caused by trichomoniasis, the remaining captive birds were re-examined for trichomonads and were found to be free of this parasite. Nodular growths were found on the heads of four doves. Small discrete, roughly diamond-shaped lesions were found in the pharynx of a fifth dove. Repeated attempts were made to isolate trichomonads from this bird, but without success. When the doves were examined again for trichomonads on October 15, one dove, which had been normal on October 5th, showed small caseous lesions on the base of the tongue. Repeated cultures for trichomonads were made from this bird during the period October 15 to October 27, when the dove died. All attempts to isolate trichomonads failed. At the time of death on October 27 there were large cutaneous lesions on both eyelids.

During the period October 6 to November 13, eleven doves

in all developed cutaneous nodular growths, and either succumbed or were sacrificed because of severe emaciation. Three of these birds showed pharyngeal lesions as well as the cutaneous nodules while the remaining eight developed only the cutaneous lesions. Although the pharyngeal lesions superficially resembled those of trichomoniasis, the cutaneous lesions and the repeated failure to demonstrate trichomonads suggested that a pox virus was responsible.

The nodular growths were removed at necropsy and fixed in 10 percent formalin. Tissues were sectioned and stained with hematoxylin and eosin and impression smears were made of the nodular material and stained with Wright's stain.

Gross and microscopic pathology. Grossly, the doves exhibited variable-sized, proliferative nodules on the eyelids, dorsum of bill and top of head. The cutaneous nodules were very numerous or few in number, ranging in size from 2 mm x 1 mm thickenings of the lower eyelid to spherical, pea-sized growths occluding the eye. The nodules appeared as small, whitish pink foci which rapidly increased in size. They became yellowish in color and coalesced to form large pea-shaped, yellow-brownish lesions. The nodules bled easily when injured. When the yellowish brown scab which had formed over the nodule was removed, a pink, seropurulent, granulating nodule was found beneath it.

The pharyngeal lesions appeared as small, yellowish, diamond-shaped areas at the base of the tongue or at the back of the pharynx and ranged from 1 mm x 1mm to 3mm x 1 mm in size. The large caseous masses typical of advanced trichomoniasis were not seen.

Microscopically, the nodules were typical of lesions in the domestic fowl. The cytoplasmic inclusion bodies (figure 1), typical of avian pox, were eosinophilic, spherical to ovoid in shape, and 8 μ to 14x20 μ in size. Impression smears stained with Wright's stain demonstrated that the large inclusion bodies contained the smaller spherical Borrel bodies. No inclusion bodies were found in the basal cell layer although small inclusion bodies were seen in cells adjacent to it. Although the cell nuclei were displaced toward the cell wall by

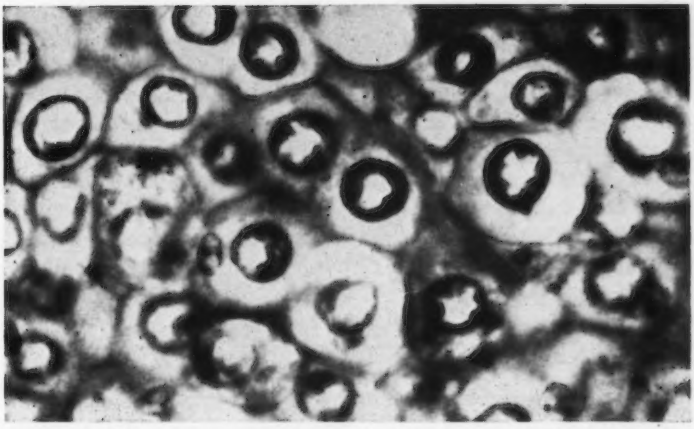

Fig. 1. Section of pox nodule from mourning dove. Cytoplasmic inclusion bodies are present. 970X.

the inclusion body, they showed no pycnotic changes.

Material from the pox lesion on the eyelid of the dove trapped on September 17 was rubbed on the conjunctiva and on the dorsum of the bill of one pigeon. A second pigeon was exposed by rubbing nodular material into an incision on the leg. Although both birds were observed for a period of one month, neither developed pox lesions. This may have been because of prior immunity or because of using a poor exposure site.

DISCUSSION

It is not known whether the pox infection in mourning doves is caused by pigeon pox or a separate strain. Kossack and Hanson[2] used commercial pigeon pox vaccine to protect their mourning doves and believed that the vaccination aided in stopping the spread of the disease.

Although avian pox usually produces nodular lesions upon eyelids, head, or legs, highly virulent pox strains may cause only caseous lesions in the oral cavity, similar to those of trichomoniasis. Stafseth[4] found that most pigeons infected

with pox showed lesions on the feet, legs, and near the base of the bill. A few birds showed cankers in the mouth.

Kossack and Hanson[2] reported a case of pox in a ring dove that developed diamond-shaped lesions in the pharynx 11 days after the appearance of the cutaneous lesions. In our series, one dove showed only the diphtheritic lesions, three doves showed cutaneous and diphtheritic lesions, and the remaining eight doves developed only the cutaneous lesions. Differentiation of pox and trichomonad lesions can be made by demonstration of the trichomonads by microscopic smear or culture. Pox can be identified by infectivity tests, or by examining smears for presence of inclusion bodies.[1]

SUMMARY

1) An outbreak of avian pox in a captive flock of mourning doves involving 12 of 23 birds is described. Typical nodular growths on the eyelids and head as well as yellowish lesions at the base of the tongue and at the back of the pharynx were seen. Typical intracytoplasmic inclusion bodies were demonstrated. Methods are described for differentiating this infection from trichomoniasis. An attempt to transmit the disease to pigeons with material from a natural case, failed.

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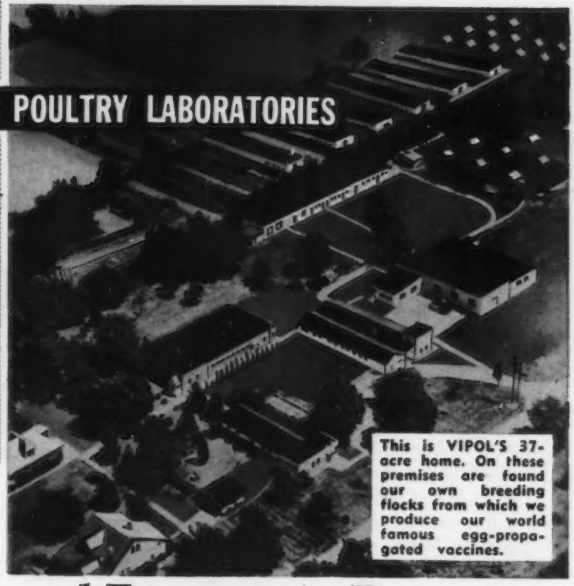

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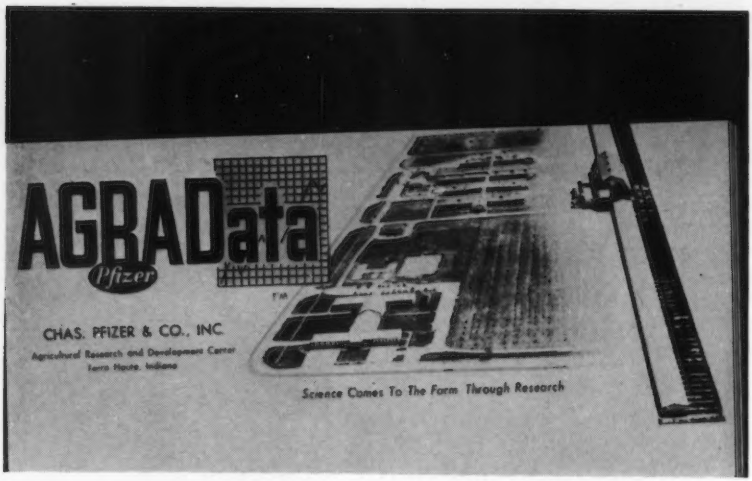

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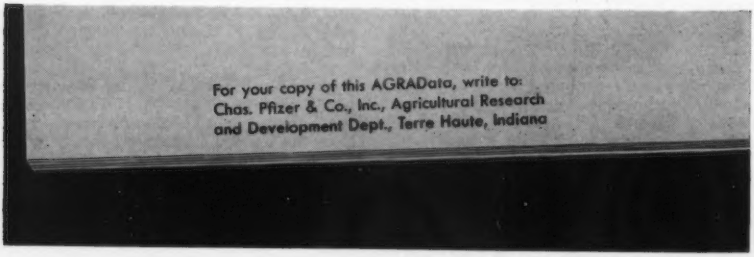
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